

**Laticifers and latex in *Papaver somniferum* L.;
capacity, development and translocation**

by

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Abstract

The opium poppy, *Papaver somniferum* L., is a pharmaceutically important species that is of commercial significance in the state of Tasmania, Australia. Poppies are grown for seed and notably for their alkaloids, including morphine, codeine and thebaine. Alkaloids accumulate in the capsule during plant development, sequestered to vesicles within the latex of the reticulated network of laticifer cells. Laticifers, as the repository for alkaloids in poppies, are potentially an important component in the yield of alkaloids, and there are aspects of these cells that have not been fully investigated. The studies herein examined the volumetric capacity of capsule laticifers; the allocation of biomass to poppy capsules; and the capability of laticifers to deliver alkaloids to the capsule through the bulk movement of latex.

Capsule laticifer capacity is of relevance in the cropping system used in Australia, where capsules are not lanced and remain intact until harvesting, which occurs after plant senescence. Variations in the volumetric capacity may represent a component of the alkaloid yield from capsules, and the first investigation assessed the quantity and variability of this parameter in field grown poppies. The latex volume was calculated for capsules that were harvested at one week, and at two weeks after flowering. It was demonstrated that the volume of laticifers per unit area of tissue was established early in capsule development (before the first week of flowering) and did not increase thereafter, yet capsule morphine content continued to rise. The capacity determined at either stage did not predict the end-yield of morphine, but there was a positive linear relationship with capsule mass, and capsule mass was the most important factor in capsule morphine yield. It was concluded that the volumetric capacity of capsule laticifers was

established early in capsule development, and therefore has limited utility as a component that may be manipulated to increase capsule alkaloid yields. Factors influencing capsule mass were further investigated.

The within-plant allocation of biomass to capsules was investigated in a field study of *P. somniferum* plant morphology. Greater than 80% of the capsule biomass was allocated to the upper three capsules, with the senescence of reproductive structures more prevalent on lower branches. The findings indicated that in the development of this commercial line of poppies there was sequential regulation of the sinks (fruit), and that the majority of reproductive biomass was allocated to the early developing capsules in terms of both fruit and seed. An argument was presented to suggest that developmental processes and within-plant resource limitation may influence the allocation of biomass to reproductive structures in poppies. Due to the complex interplay of sink strength and feedback mechanisms, and with so little modelling of within-plant biomass allocation in poppies, additional studies were suggested.

The capability of laticifers to deliver alkaloids to the capsule through the bulk movement of latex was conducted through an investigation of the vesicle populations and osmotic potential of both capsule latex and stem latex in *P. somniferum*. Vesicle populations were examined during plant development, at localised positions and in terms of their volume. Capsule latex contained significantly greater vesicle numbers, packed vesicle volume (PVV), and osmotic potential than stem latex, and these significant differences occurred as a steep gradient across the thalamus of the poppy capsule. Latex from three sampled positions on plants of *P. somniferum* also differed in the development of vesicle populations, with capsule latex exhibiting a steep increase in

vesicle number after flowering. The findings indicated that the development of latex (within laticifers) was localised to separate organs, and that *in toto* latex movement to the capsule was unlikely.

In summary, the research in this thesis reveals that laticifers represent a consistent volume in capsule tissue; that capsule mass is important for alkaloid yield and that the upper capsules have an advantage in the development of that mass; and that the *in toto* movement of latex through laticifers into the capsule is improbable.

Abbreviations

ASD	apical stem diameter
BBE	berberine bridge enzyme
BSD	basal stem diameter
CA	capsule morphine content
CI	confidence interval
CNMT	coclaurine <i>N</i> -methyltransferase
COR	codeinone reductase
CPS	concentrate of poppy straw
CYP80B1	(<i>S</i>)- <i>N</i> -methylcoclaurine 3'-hydroxylase
DF	degrees of freedom
DW	dry weight
ER	endoplasmic reticulum
f+3	sampling stage at three days after flowering
f+7	sampling stage at seven days after flowering
f+11	sampling stage at eleven days after flowering
f+14	sampling stage at fourteen days after flowering
GLM	general linear model
HPLC	high performance liquid chromatography
L	exuded latex mass
LA	exuded latex morphine content
LM	total latex mass (calculated latex mass in capsule at sampling)
MLP	major latex protein
NMR	nuclear magnetic resonance
PVV	packed vesicle volume
SAT	salutaridinol 7- <i>O</i> -acetyltransferase
SE	standard error
SEM	standard error of the mean
SDS	sodium dodecyl sulphate
TYDC	tyrosine decarboxylase

ψ_s	osmotic potential
4 OMT	3-hydroxy- <i>N</i> -methylcoclaurine 4- <i>O</i> -methyltransferase
6 OMT	norcoclaurine 6- <i>O</i> -methyltransferase
7 OMT	reticuline 7- <i>O</i> -methyltransferase

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CHAPTER 1 General introduction and literature review

1.1 Commercial production of *Papaver somniferum* L. in Tasmania, Australia

The opium poppy plant, *Papaver somniferum* L., is legally grown in several countries for pharmaceutical products (analgesics, anti-tussives) and for culinary purposes (seeds, oil). In the southern hemisphere, commercial production is limited to the state of Tasmania, Australia, where the industry has considerable economic importance, with a farm-gate value of \$65 million and an overall contribution of over \$200 million annually to the state's economy (Department of Primary Industries, Water and Environment, Tasmania 2005). Tasmanian production of this unique crop has expanded since it began in the 1960s and it is a valuable agricultural industry in the state, dominating the pasture, cereal and field crop sectors (Laughlin *et al.* 1998). Production varies yearly according to the demands of the pharmaceutical market, but as a consequence of the standing 80/20 rule (United States Department of Justice, Drug Enforcement Administration 2006) which governs global supply locations, Tasmania consistently supplies 40% of the world's licit opiates and also exports seed worldwide (Millgate *et al.* 2004).

In Tasmania the poppy plant is grown to full dry maturity before harvesting occurs, unlike the production in India, where capsule latex (opium) is collected over the entire growing period. Tasmanian commercial producers mechanically harvest the capsules and a small portion of the upper stem at dry maturity, the seeds are removed and the remaining material (straw) is ground and extracted for alkaloids; secondary metabolites.

It is these alkaloids, including morphine, codeine and thebaine, sourced from the concentrate of poppy straw (CPS), that confer pharmacological properties to the plant, and in the mature poppy plant these accumulate in the fruit (capsule), in a specialised secretory system of cells termed 'laticifers'.

1.2 Project background

The poppy industry is highly competitive and there is an imperative to make continual advancements in the yield of alkaloids in order to maintain the standing of the Tasmanian industry in the world market. Current research predominantly focuses on the agro-morphological, biochemical and molecular aspects of morphine production (Prajapati *et al.* 2002, Ziegler *et al.* 2008) and recent work has notably resulted in the development of the thebaine poppy, which has considerable economic importance as it does not fall under the purview of the 80/20 rule (Fist 2001, Millgate *et al.* 2004).

With research focussing on other areas of alkaloid production and yield, the role of laticifers in the yield of alkaloids has not been thoroughly addressed. Since the early anatomical work of Fairbairn and Kapoor (1960), the extensive ultrastructural investigations of Nessler and Mahlberg (1977a, 1977b, 1978, 1979i, 1979ii, 1981), and the more recent cellular localisation studies which have investigated the role of other cell types in alkaloid biosynthesis (Bird *et al.* 2003, El-Ahmady and Nessler 2001, Facchini and De Luca 1995, Samanani *et al.* 2006, Weid *et al.* 2004), there have been few studies specifically targeting laticifers, and thus it is an area that could be extended.

To this end, this research investigated two aspects of laticifers that may prove significant to alkaloid yield and which have not been adequately studied; the physical capacity (volume) of capsule laticifers, and the capability for functional latex movement within the laticifer network.

1.3 The storage site of alkaloids – laticifers

Laticifers, or latex vessels, are the secretory cells found in species of plants that contain latex. Species such as rubber (*Hevea brasiliensis*), spurge (*Euphorbia sp.*), milkweed (*Asclepias sp.*) and poppies are just a few of the 20 000 species that are latex-bearing (Agrawal and Konno 2009). Laticifers are the specialised repositories for secondary metabolites, and in the case of poppy, they are the ultimate destination of alkaloids, which are sequestered to vesicles within these cells (Fairbairn and Djoté 1970, Fairbairn *et al.* 1974, Roberts *et al.* 1983).

Laticifers are located throughout the entire poppy plant including all floral parts, but the most extensive network of these cells develops in the capsule pericarp (El Kheir 1975, Fairbairn and Kapoor 1960, Kapoor 1995, Nessler and Mahlberg 1976). The laticifers of *Papaver somniferum* L. are both articulated and anastomosing, forming a highly reticulated cellular network through dissolution of cell walls (Nessler and Mahlberg 1977a, 1977b, 1981). Research indicates that variations exist both in the anatomy of the laticifer system, and in the yield of latex from laticifers. Anatomical differences have been noted between subspecies in terms of both the number of cells and the amount of reticulation between cells in the capsule wall (Veselovskaya 1976), and several authors have noted differences in opium yield of *Papaver somniferum* L. (Pareek *et al.* 1995,

Wang *et al.* 1999). It is possible therefore, that variation in the number and connectivity of laticifers (that is, the volume or capacity of the repository), may be a component in explaining variation in alkaloid yield, but as yet there has been no research that addresses the physical capacity of capsule laticifers as a yield component of alkaloids in CPS production of *Papaver somniferum* L.

1.4 Laticifer development and reticulation

The capacity of the system of laticifers is determined through the development and connectivity of the cells. Laticifers are located within the vasculature of the plant, proximal to phloem cells but not associated with xylem (Datta and Iqbal 1994, Fairbairn and Kapoor 1960, Thureson-Klein 1970). They are initiated by the vascular cambium and have been identified in the early stages of plant development, just days after germination (72h) and coincident with the differentiation of the procambium. The connections between cells occur through the enzymatic dissolution of the cell walls, late in cell differentiation (Nessler and Mahlberg 1977b, Pilatzke-Wunderlich and Nessler 2001). Cellulase and several pectinases have been identified as the enzymes responsible for the process, with dissolution occurring late in development from both sides of the lamella in transverse walls, but in lateral walls it occurs at specific sites (Nessler and Mahlberg 1977b, 1981). It has also been claimed that laticifers in poppy have plasmodesmatal connections with neighbouring sieve cells (Facchini *et al.* 2007, Facchini and De Luca 2008).

1.5 The contents of laticifers: latex

Studies have established that poppy laticifers are the repository for alkaloids, and anatomical investigations have observed and described the connections between these cells throughout the plant, but do laticifers have functions other than storage?

Laticifers contain latex, a complex suspension of an array of substances such as mucilages, carbohydrates, enzymes, amino acids, proteins and products of secondary metabolism. Latex also contains structural components such as alkaloid vesicles, lipid bodies and the usual component of cytosolic organelles, e.g. nucleus, endoplasmic reticulum and mitochondria (Datta and Iqbal 1994, Thureson-Klein 1970). Further, latex is by no means biologically inactive. Several authors have established that both metabolic processes and enzymatic reactions occur within latex (Decker *et al.* 2000, Roberts *et al.* 1983), and Weid *et al.* (2004) identified that the later stages of morphine biosynthesis occurs in the latex by establishing that codeine reductase (COR; catalyses codeinone-codeine and morphinone-morphine, Appendix 1) was localised to laticifers.

Aside from the functions of storage and synthesis, laticifers have also been attributed the function of alkaloid translocation by the transport of substances through the reticulated system. Fairbairn *et al.* (1974) in their study of translocation suggested that it occurred partially through *in toto* latex movement, and partially within latex via alkaloid vesicle movement, however the mechanism for either process was not elucidated. Since their study, translocation has been widely noted in the literature (Facchini and Bird 1998, Facchini and De Luca 1995, Hofman and Menary 1984, Shukla *et al.* 2001), yet research presenting direct evidence is scarce, and although

laticifers and latex synthetic ability have been investigated, translocation appears to have been overlooked. Translocation is an important aspect of accumulation because alkaloids gather in the capsule as the plant develops; so better understanding the delivery of alkaloids to the capsule may have direct implications for the yield of capsule alkaloids.

1.6 Latex translocation

There are several lines of indirect evidence that support the occurrence of alkaloid translocation in poppy; developmental patterns of alkaloid accumulation, the localisation of enzymes, latex exudation studies, and some authors have performed designated translocation studies. Certainly, with connectivity observed in anatomical studies of laticifers, the physical pathway that would allow translocation appears to be present.

1.6.1 *Developmental patterns of alkaloid accumulation*

Early studies observed a decrease in poppy leaf alkaloids after technical maturity, and Nikonov (1958) suggested that this was the result of ‘migration’ to other organs (capsule) that continue to increase in concentration. Later, Williams and Ellis (1989) found that in younger plants (40 to 50 days old), there was a decrease in morphine and codeine concentrations in root tissue (not the latex) but that concentrations of both increased in the aerial tissues of the plant. Frick *et al.* (2005) also observed that morphine concentration was greatest at the apex of the plant and decreased toward the roots, but in contrast to Williams and Ellis (1989), codeine concentration showed the inverse pattern. Further to this discrepancy, Bajpai *et al.* (2001) found no relationship between the ratios of codeine to morphine in the pedicel versus that in the capsule, and suggested that there may be differential synthesis of alkaloid in these structures. Although some of the research presents conflicting observations, there are indications that during development the products of the later stages of alkaloid biosynthesis accumulate in the plant apex, and importantly, the possibility of transport is not necessarily precluded.

1.6.2 *Enzyme activity and localisation*

There have been several studies that indicate differential enzyme activity and localisation of enzymes to particular regions of the *P.somniferum* plant. An enzyme involved in the early part of the alkaloid biosynthetic pathway, tyrosine decarboxylase (TYDC), showed greater activity in the stems and roots of poppy plants than in the carpel (Facchini and De Luca 1994), and was also detected in the roots and petioles of young leaves by El-Ahmady and Nessler (2001).

Gene transcripts for enzymes further along the biosynthetic pathway, (*S*)-*N*-methylcoclaurine 3'-hydroxylase (CYP80B1) and codeinone reductase (COR), have been detected in the root, stem, leaf (lamina and midrib), bud and capsule, however the relative expression of transcripts for both was higher in the stem than in the capsule (Huang and Kutchan 2000). Further, Gerardy and Zenk (1993a, 1993b) demonstrated that the activity of enzymes involved in catalysing the steps to salutaridine and salutaridinol (on the morphinan pathway) was higher in stems and roots but lower in capsules, also implicating upward transport of pathway intermediates.

Berberine bridge enzyme (BBE; sanguinarine pathway) transcripts and enzyme activity have frequently been detected in the stem of poppies (Facchini *et al.* 1996, Huang and Kutchan 2000, Steffens *et al.* 1985), although not by all authors (Weid *et al.* 2004). This pathway results in the production of the sanguinarine, an anti-microbial alkaloid that accumulates in the root cortex idioblasts of *P. somniferum* (Bock *et al.* 2002). The presence of BBE transcripts and enzyme activity in the shoot has been offered as evidence that alkaloid pathway intermediates are also transported to the roots, and thus the implication is that alkaloid translocation (although of different pathways) occurs in both directions (Facchini 2001).

1.6.3 Latex exudation studies

Latex exudation studies have attempted to demonstrate transport. Annett (1920) described the successive decreases in the yield of latex morphine when a poppy capsule was repeatedly lanced, and much later Fairbairn *et al.* (1974) found that successive samples of latex taken from cut stems yielded relatively consistent concentrations of morphine. This may indicate that capsule latex becomes diluted with stem latex with

continual exudation, and that stem latex is relatively consistent in content (but lower in alkaloid concentration than the capsule). Indeed, Tookey *et al.* (1976), stated that lancing does not change the overall plant alkaloid content, but that lancing increased the yield from the capsule and that the alkaloids translocated upward were not replaced in the stem.

These studies illustrate the capacity for latex exudation under vascular trauma; exudation which occurs due to the turgor pressure of laticifers. They also demonstrate the effect of lancing on alkaloid concentration in exuded latex, but they do not provide evidence for, or an explanation of, the occurrence of substance movement in an undisturbed laticifer system. To this end some researchers have performed designated translocation studies.

1.6.4 Translocation studies

Fairbairn and El-Masry (1967), Fairbairn *et al.* (1964) and Fairbairn *et al.* (1974) supplied radio-labelled morphine to peeled stems of poppy plants and were able to detect the presence of (varying) levels in the capsule tissue and latex. Vágújfalvi (1966) also performed translocation experiments on seven latex bearing species including *P. somniferum*, *Euphorbia falcata* and *Sonchus oleracea*, applying radio-labelled codeine to leaves, and found that there were greater amounts in the stem latex above the leaf than below. The research appears to indicate that transport had occurred in latex, yet there was no elucidation of the process that may have led to the appearance of radio-labelled alkaloid in other plant parts. This is of note, especially as in the work of Fairbairn *et al.* (1974), whilst some radioactivity was detected in exuded capsule latex (15-23% of total applied), there were considerable quantities located in both the

pericarp tissue (54-63%) and the ovules (20-31%). Also, the study of Fairbairn and El-Masry (1967) detected 36% of fed radioactivity in the capsule, 54% in the stem and 9% in the leaves, with a small proportion in the roots. The possibility of contamination must be considered, especially in light of the coarse method of introducing the radio-labelled morphine, and the fact that their previous stem application had resulted in only 70% of radioactivity being present in the vesicle fraction of the stem (so the presumption that morphine is entirely sequestered to vesicles within the latex was not fully supported). The authors did address this concern and discussed the possibility of leakage from vesicles, but the alternate explanation is that perhaps the labelled morphine was not taken up in the first instance.

Further to this, at the cellular level Facchini *et al.* (2007) describe the involvement of other cell types in the alkaloid biosynthetic pathway. Although there is some contention regarding the particular cellular sites of the biosynthetic pathway, many authors have illustrated the involvement of phloem parenchyma, sieve cells and companion cells in the synthesis of alkaloids (Bird *et al.* 2003, Samanani *et al.* 2006, Weid *et al.* 2004).

Thus, with the combination of experimental inference, possible experimental contamination, and evidence demonstrating the transport of alkaloids in both xylem and phloem of other latex-bearing genera (De Luca and St-Pierre 2000, Wink and Roberts 1998), other mechanisms of translocation (aside from the system of laticifers) cannot be discounted.

It is evident therefore, that while there are many references in the literature to alkaloid translocation in poppy laticifers, there is little research that has clearly and robustly

demonstrated the capability of, and mechanism for, substance transport within this system (Pickard 2008). Research has not been able to adequately address the possibility of movement in a contained system where there is no release of pressure from these cells.

1.7 Research aims

This research sought to examine the aspects of laticifers (volume, capacity, content, translocation) that may contribute to alkaloid yield in poppy capsules of commercial varieties of *P. somniferum*.

Chapter 2 investigated the significance of laticifer capacity (volume) in the alkaloid yield of capsules. It was hypothesised that the capacity of laticifers in the capsule contributes to the yield of morphine from a capsule.

Chapter 3 investigated the within-plant biomass allocation to capsules. It was hypothesised that the allocation of dry matter to capsules was dependent on plant architecture.

Chapter 4 investigated the utility of nuclear magnetic resonance (NMR) in finding evidence for the functional movement of substances in the undisturbed laticifer system. It was hypothesised that laticifers have the capability, through extensive articulation, for the bulk translocation of latex from the stem to the capsule.

Chapters 5 and 6 also investigated the evidence for the functional movement of latex in the undisturbed laticifer system. It was hypothesised that there would be a relationship between latex characteristics in the stem and the capsule.

CHAPTER 2 The capacity of laticifers in capsules of poppy, *Papaver somniferum* L. *

2.1 Abstract

This study found that the latex capacity (mg latex/mg dry weight capsule) of opium poppy capsules is fixed early in capsule development. Latex capacity, which represents the proportion of the capsule wall allocated to laticifers (specialised cells for latex storage), had peaked in the capsule at one week after flowering. In contrast, the morphine content of capsules continued to increase with capsule development until commercial harvest. Morphine content was correlated with capsule mass and total latex mass, but there was no correlation between latex capacity and morphine yield. The most important morphological characteristic in terms of morphine end-yield (commercial harvest stage) was capsule mass. The findings of this study demonstrate that although latex yield per plant is a highly heritable morphological characteristic (Jain *et al.* 2005, Yadav *et al.* 2006), it may have limited potential for use in a breeding strategy aimed at increasing the morphine yield from capsules.

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2.2 Introduction

The state of Tasmania, Australia, supplies up to 40% of the world's licit opiates which are predominantly exported to the USA for use in pharmaceuticals. Since the production costs for poppy (*Papaver somniferum* L.) are high in Tasmania, there is an imperative to work toward improvements in alkaloid yield from field crops so that the state's producers remain competitive in this industry.

Many factors have previously been identified as important in the yield of alkaloids, and positive correlations have been identified between the yield of morphine and the number of capsules per plant, and the mass of capsule husk (Mika 1955). There have also been extensive investigations into the relationship between alkaloid yield, opium yield (dried latex) and morphological characteristics (Singh *et al.* 2003, Srivastava and Sharma 1987, Yadav *et al.* 2006). Several authors report positive correlations between opium yield and morphological characteristics including; stem diameter, capsule size and the number of capsules per plant (Yadav *et al.* 2004), and negative correlations between opium yield and the morphine content of opium (Bhandari *et al.* 1997, Singh *et al.* 2000, Singh *et al.* 2003, Yadav *et al.* 2006).

The majority of studies on alkaloid yield components are based on a traditional repeated-lancing harvesting technique for the collection of latex from capsules. As this is not performed in Australia, it is difficult to extend the conclusions to a production system where latex is not removed from capsules and where plants are left intact until harvesting, which occurs after senescence. However, since the latex of poppies is both the site of alkaloid accumulation and a morphological characteristic (latex yield per

plant) with high heritability and high genetic gain, it has potential as a suitable characteristic for consideration in breeding strategies to increase alkaloid yields (Jain *et al.* 2005, Yadav *et al.* 2006).

Latex is exclusively contained in a secretory system of laticifer cells that develop as an extensively branched network throughout the poppy plant, but particularly within the capsule wall (Fairbairn and Kapoor 1960). Reticulation of the laticifer system is described as being fully developed at two weeks after flowering, and this is the stage at which both capsule size and the mass of morphine are reported to be greatest (Kapoor 1995, Annett 1920, Mika 1955). It could be postulated that this would also be the stage where capsule latex capacity (mg latex/mg dry weight (DW) capsule) would be greatest, as the cellular infrastructure (laticifer network) is fully developed.

There is some contention regarding the role of laticifers in the synthesis of alkaloids, with researchers presenting evidence for the involvement of other cell types in the aerial organs of the poppy plant, such as phloem parenchyma (Weid *et al.* 2004). Bird *et al.* (2003) and Samanani *et al.* (2006) claim that the spatial distribution of synthesis extends to the sieve cells of phloem, and that the co-localisation of alkaloid biosynthetic enzymes occurs in all poppy organs as the plant develops. This observation may also lend support to the argument that alkaloids are translocated from other organs of the poppy plant to the capsule, raised previously by several authors (Facchini and Bird 1998, Gerardy and Zenk 1993a). The concept of translocation, however, is not universally supported. For example, research by Bajpai *et al.* (2001) argues for a differential regulation of alkaloid synthesis between capsule and peduncle, based on their observation that the accumulation of codeine and morphine between peduncles and

capsules was independent. It is evident then that uncertainty remains in terms of the location of alkaloid synthesis and the occurrence of translocation, but regardless of whether alkaloids are synthesised in the capsule or translocated into the capsule, it is possible that the latex capacity of the capsule (as the ultimate storage place for morphinan alkaloids) is an important determinant of the alkaloid yield.

As yet, there have been no studies addressing whether the latex capacity of an intact capsule is related to the end-yield of alkaloid (i.e. at commercial harvest stage). The main objectives of this study were to:

1. develop a method for calculating the latex capacity of an intact capsule
2. calculate the latex capacity of capsules at two stages of development, and
3. investigate the relative importance of latex capacity amongst other morphological characteristics in the end-yield of morphine in a capsule.

2.3 Materials and methods

2.3.1 *Experimental sites and design*

Capsule samples of a high morphine producing line of *P. somniferum* were obtained from several commercial crop sites in Tasmania during the southern hemisphere summer of 2005/2006. An extensive investigation was performed with material from four sites in the Coal River Valley in southern Tasmania. For each site plant density was calculated, capsule latex was sampled at two stages, and at a third sampling stage a number of plants were morphologically assessed.

In addition to the four southern Tasmanian sites, three supplementary sites from the Deloraine area in northern Tasmania were chosen, to broaden the applicability of the study. These sites were established from a different seed lot of the same variety and were planted later in the season. Capsule samples at these sites were taken on two occasions to be sampled for latex.

Within each of these seven sites (southern and northern), four random plots were allocated. On the same day, twenty plants were tagged in each plot, with each plant consisting of a main branch with two lateral branches. Only plants that had flowered on that day were selected. At one week and then again at two weeks after flowering, ten capsules were collected from each plot. The first (uppermost) lateral capsule was taken for analysis at all sites.

Of the four southern sites, site 1 (as the pilot site) was tagged and sampled four days earlier than the other southern sites, but the time of day at which sampling occurred and

the stage (e.g. flowering) was consistent across all sites. The collection of capsules from the other three southern sites was co-ordinated so that all were sampled simultaneously in the morning. This was done to circumvent issues of diurnal fluctuations in latex water content between sites (Itenov *et al.* 1999), and thus relative alkaloid yield. The three supplementary sites in the north were sampled sequentially (early in the morning).

2.3.2 Sampling at week one and at week two after flowering

At both week one and week two after flowering, latex and capsules were sampled and processed. In the field, the plant stem was gently heated 15 cm below the capsule to coagulate the latex inside the stem (Spencer 1939b). The stem was cut at this point, the sample bagged and placed in the dark on ice for transport. To process samples, the plants were cut just below the thalamus of the capsule and the immediate exudation of latex was collected into pre-weighed 1.5 ml Eppendorf vials containing 1 mL of sodium dodecyl sulphate buffer (2.5% SDS in 20% ethanol and 2.3 gL⁻¹ NH₄H₂PO₄ buffer). Once exudation ceased, the capsule was immediately frozen in liquid nitrogen. Capsules were freeze-dried, the seeds were removed, and the remaining capsule wall was extracted and assayed for morphine content using an isocratic high performance liquid chromatography (HPLC) method developed primarily for the identification and quantification of morphine. The latex collected into the buffer was weighed and also assayed for morphine content using a Waters Alliance HPLC (Alltech Platinum C18, 3 µm, Rocket Column; wavelength 254 nm; column temperature 40°C; pump speed 1.5 mL min⁻¹; injection volume 15.0 µL; and water:acetonitrile gradient ranging from 2% to 32% acetonitrile over 18 min).

2.3.3 Latex mass calculation

This trial employed a calculation method to assess the total latex mass of poppy capsules at two stages of capsule development. The total amount of latex contained in the capsule was calculated using the mass of exuded latex, the morphine content of that latex, and the morphine content of that capsule (containing the remaining latex) *viz*:

$$LM = \left(\frac{CA}{LA} \right) \times L + L$$

Where:

LM - total latex mass (calculated latex mass in capsule at sampling)

CA - capsule morphine content

LA - exuded latex morphine content

L - exuded latex mass

Using the ratio of capsule morphine to latex morphine assumes that the morphine in a capsule is contained wholly in the latex; an assumption extensively supported by other researchers (Facchini *et al.* 2007, El-Ahmady and Nessler 2001, Kutchan *et al.* 1985). The calculation also assumes that morphine concentration is consistent throughout the latex in the capsule. Uniformity of morphine distribution was confirmed in a glasshouse investigation prior to the field study (data not shown).

2.3.4 Sampling at stage three, plant senescence (dry maturity)

Mature *P. somniferum* plants were collected from the four southern sites in Tasmania during the summer season of 2005/2006. At each site, four plots were marked out at randomly selected locations and plants were sampled from these positions. A total of

20 plants were removed from within each of these four plots. Plant density was calculated in each plot by counting the number of plants in 1 m².

Sampled plants were measured for height, number of nodes, number of branches, stem mass and for several capsule traits. These included capsule position on the plant, height, dry mass, capsule diameter and the number of stigmatic rays. Latex mass was not assessed since at stage three (dry maturity), the plants had senesced and liquid latex was not present in the capsule.

From a subset of plants, screened on the basis of architecture (three branches, each terminating in a capsule), all capsules were individually processed by removing seeds, then grinding and extracting the capsule wall for alkaloids. Morphine content was assayed using HPLC.

2.3.5 Data analysis

A Pearson's two-tailed correlation was performed on capsule data for both sample stages using the statistics package SPSS v14.0. A bivariate analysis using ASReml was conducted to ascertain the relationship between latex capacity and final morphine yield. Finally, the dataset from the morphological investigation conducted at final sampling was analysed by stepwise linear multiple regression, using SPSS v14.0. Mean values are presented with standard error (SE) bars.

2.4 Results

At the first sampling (one week after flowering) the mean LM per capsule ranged from 319 mg (site 1) to 487 mg per (site 2). Mean dry capsule mass ranged from 1024 mg (site 4) to 1357 mg (site 2) and mean morphine mass in the capsule ranged from 12 mg (site 1) to 21 mg (site 2; Figure 2.1).

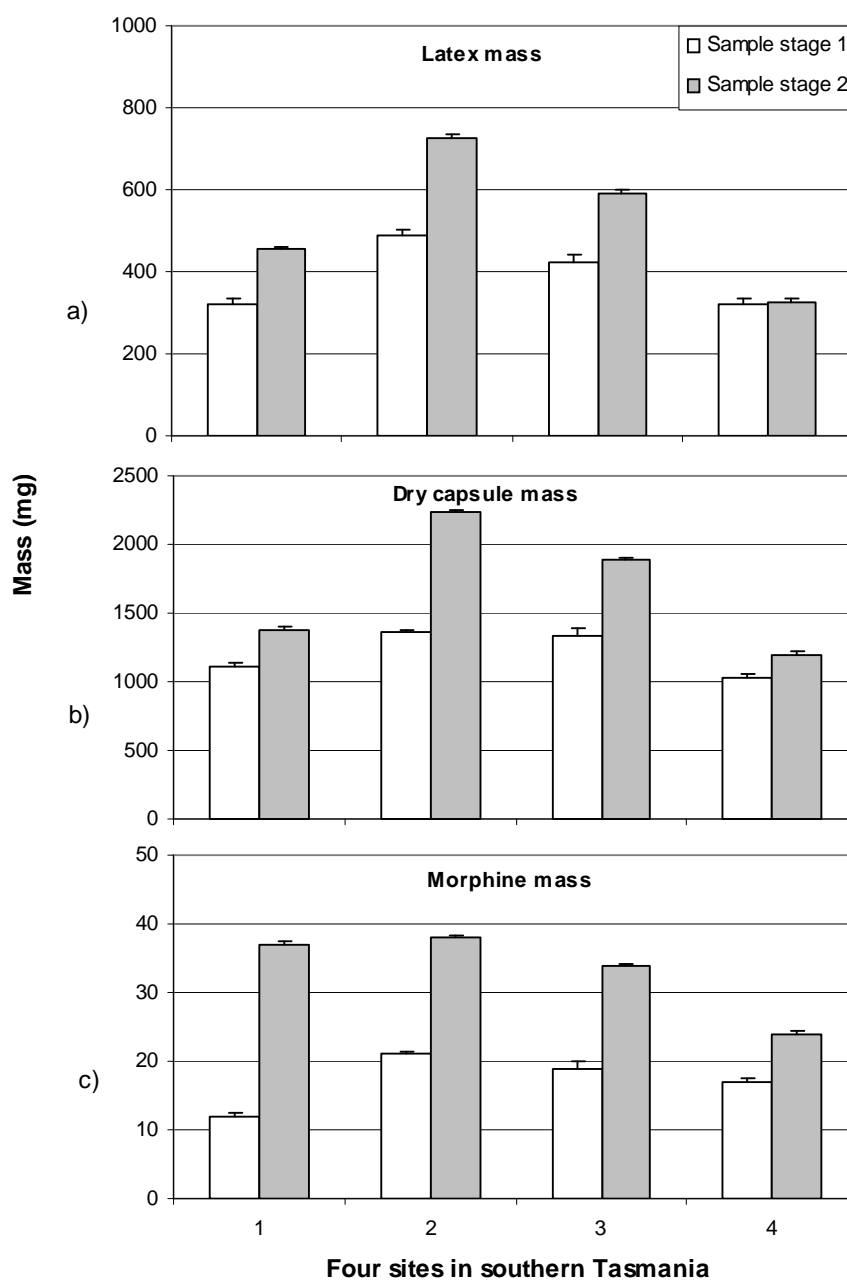


Figure 2.1. Mean site values for a) latex mass (LM), b) dry capsule mass, and c) morphine mass for sample stages 1 and 2, respectively one week and two weeks after flowering. $LM = (CA/LA) * L + L$, where: LM = the calculated original latex mass in capsule, CA = the measured capsule morphine content, LA = the measured latex morphine content and L = the latex mass. Upper SE bars shown, 1 SEM, n = 10.

At the second sampling (two weeks after flowering), the mean LM had increased on a per-capsule basis, and ranged from 325 mg (site 4) to 727 mg (site 2). There was also an increase in both the mean dry capsule mass and the mean morphine mass per capsule. Dry capsule mass ranged from 1191 mg (site 4) to 2230 mg (site 2). Morphine mass in the capsule at this stage ranged from 24 mg (site 4) to 38 mg (site 2; Figure 2.1).

Although on a per-capsule basis there was an increase in the LM, the dry capsule mass, and the morphine mass from sample stage 1 to sample stage 2, this was not the case for sample stage 2 to sample stage 3 (dry maturity). The mean dry capsule mass either remained the same (site 1) or decreased by as much as 20% (site 2), yet the mean morphine mass continued to increase in the capsule (at all sites) by up to 35% (site 2). The mean latex mass was not assessed at stage 3 as the plants had senesced and (liquid) latex was not present for sampling.

A Pearson's two-tailed correlation revealed strong positive relationships at sample stages 1 and 2, between dry capsule mass and total morphine mass in the capsule ($r = 0.85$), between dry capsule mass and latex mass ($r = 0.89$) and between dry capsule mass and latex exudate at the time of sampling from the capsule ($r = 0.78$). Moderate and positive correlations were apparent between the number of stigmatic rays on a capsule and the dry capsule mass ($r = 0.42$), and the number of stigmatic rays and calculated latex mass ($r = 0.49$). These correlations describe linear trends and are significant at $P = 0.0001$ (Table 1.1). Additionally, there was a moderately negative, highly significant relationship between the amount of exuded latex and the concentration of morphine in that latex ($r = -0.52$, $P = 0.0001$), i.e. when there was a greater amount of latex exudate from a capsule, the morphine concentration was lower.

Table 1.1. Correlation matrix of variables measured at sample stages 1 and 2, one week and two weeks after flowering respectively, from samples collected from seven experimental sites.

Variables	Dry capsule mass (g)	Exuded latex (g)	Latex morphine (%)	Stigmatic rays (no.)	Latex mass (g)	Total mass morphine (g)
Sample stage	$r = 0.31$ $P = 0.000$	$r = 0.23$ $P = 0.002$	$r = 0.11$ $P = 0.127$	$r = 0.09$ $P = 0.202$	$r = 0.35$ $P = 0.000$	$r = 0.60$ $P = 0.000$
Dry cap mass (g)		$r = 0.78$ $P = 0.000$	$r = -0.24$ $P = 0.002$	$r = 0.42$ $P = 0.000$	$r = 0.89$ $P = 0.000$	$r = 0.85$ $P = 0.000$
Exuded latex (g)			$r = -0.52$ $P = 0.000$	$r = 0.46$ $P = 0.000$	$r = 0.85$ $P = 0.000$	$r = 0.61$ $P = 0.000$
Latex morphine (%)				$r = -0.28$ $P = 0.000$	$r = -0.43$ $P = 0.000$	$r = -0.10$ $P = 0.163$
Stigmatic rays (no.)					$r = 0.49$ $P = 0.000$	$r = 0.40$ $P = 0.000$
Latex mass (g)						$r = 0.87$ $P = 0.000$

Although total latex mass in the capsule increased from stage 1 to stage 2, the latex mass per unit tissue (mg latex/mg DW capsule, or latex capacity) did not increase over time. Latex capacity was assessed for capsules sampled in the extensive study of the four southern Tasmanian sites (Figure 2.2a), and it was evident that latex capacity had reached the maximum value by the first sampling stage (one week after flowering). While latex capacity in capsules from these sites had peaked by stage 1, morphine concentration continued to increase beyond stage 1 until the final sample stage (Figure 2.2b). All sites demonstrated an increasing trend in morphine concentration as sampling advanced.

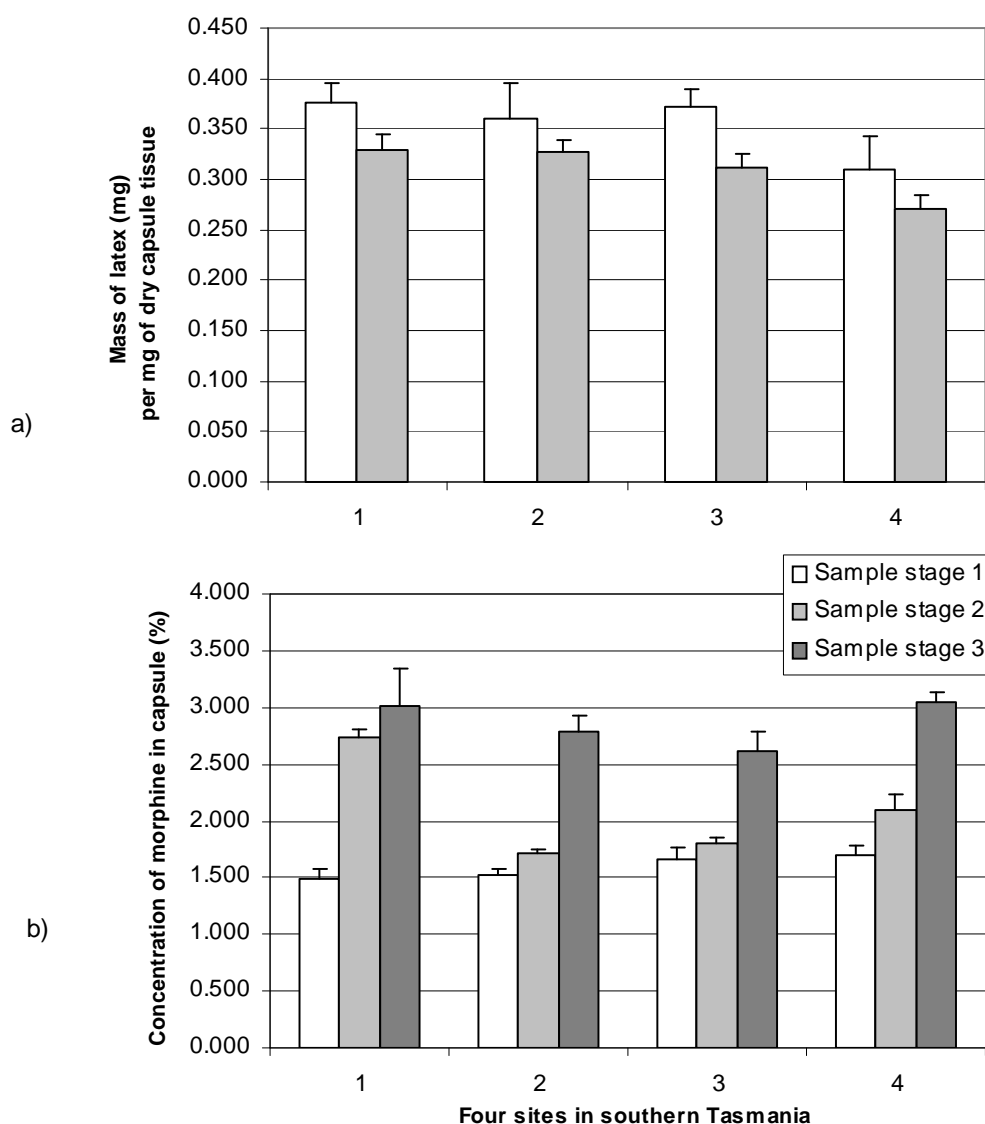


Figure 2.2. Site averages for a) latex capacity and b) morphine concentration, from sites sampled in Tasmania. Latex capacity is the total mass of latex (mg) expressed per mg dry capsule mass (of that particular capsule). Morphine concentration is the total mass of morphine in a capsule (mg) per mg of dry capsule mass of that capsule, expressed as %. Sample stages are one week after flowering, two weeks after flowering and at dry maturity, respectively. Upper SE bars shown, 1 SEM, $n = 10$.

The laticiferous system is the site of alkaloid accumulation in the poppy capsule, so although the timing of development of the laticifers differed from that of morphine accumulation, it was hypothesised that the capacity of the laticifers set early in capsule development may influence alkaloid accumulation later in capsule development.

Therefore, a bivariate analysis was conducted using ASReml to ascertain whether there was a relationship between the latex capacity at either sample stage 1 or sample stage 2 (Figure 2.2a), and the final morphine yield from sample stage 3. At sample stage 1, there was a moderate positive correlation between latex capacity and the morphine yield at sample stage 3, but it was not significant ($r = 0.40$; $P = 0.17$). At sample stage 2, there was no correlation between latex capacity and the morphine yield at sample stage 3 ($r = 0.015$, $P = 0.18$). Correlations with other alkaloids (such as codeine and thebaine) were not investigated as in high-yielding morphine lines they represent only a minor proportion of the total alkaloid yield at commercial harvest stage (Hofman and Menary 1984).

Finally, the stepwise regression to assess the relationship between the morphological characters and morphine yield measured at stage 3 (Table 2.1), indicated that dry capsule mass was the best predictor of the morphine concentration within that capsule ($R^2 0.86$, $P < 0.0001$).

Table 2.1. Mean site density and mean site values with \pm SE, of morphological variables measured at sample stage 3, $n = 10$ for each of the 4 experimental sites in southern Tasmania. BSD = basal stem diameter and ASD = apical stem diameter.

Southern sites	1	2	3	4
Site density (plants/m ²)	49.17 \pm 4.98	33.44 \pm 5.30	28.94 \pm 3.57	34.67 \pm 3.65
Height (cm)	110.20 \pm 2.22	114.80 \pm 2.97	118.70 \pm 2.77	103.20 \pm 2.82
No. of nodes	18.20 \pm 0.53	17.50 \pm 0.45	16.80 \pm 0.53	17.00 \pm 0.61
BSD average (mm)	10.85 \pm 0.47	11.53 \pm 0.37	11.67 \pm 0.44	11.31 \pm 0.42
ASD average (mm)	5.44 \pm 0.10	5.58 \pm 0.09	5.69 \pm 0.10	5.08 \pm 0.13
Stem mass (g)	11.03 \pm 0.90	16.13 \pm 2.14	11.97 \pm 0.93	8.16 \pm 0.61
No. stigmatic rays per capsule	12.00 \pm 0.33	12.40 \pm 0.16	12.80 \pm 0.29	11.30 \pm 0.52
Capsule height (mm)	43.17 \pm 1.89	48.89 \pm 1.00	46.24 \pm 1.10	42.73 \pm 1.87
Capsule diameter (mm)	27.81 \pm 1.84	32.51 \pm 0.60	29.99 \pm 0.82	24.60 \pm 1.65
Dry capsule mass (g)	1.39 \pm 0.13	1.81 \pm 0.11	1.61 \pm 0.10	1.03 \pm 0.12
Morphine mass in capsule (g)	0.04 \pm 0.01	0.05 \pm 0.00	0.04 \pm 0.00	0.03 \pm 0.00

2.5 Discussion

Positive linear relationships were evident between capsule dry matter, capsule morphine mass, latex mass, and the number of stigmatic rays (Table 1.1). These correlations indicate that larger capsules have a greater mass of latex, a greater mass of morphine and a greater number of stigmatic rays, which confirms results of previous capsule-based studies (Singh *et al.* 2003). It could be posited that the presence of more stigmatic rays on larger capsules reflects the supporting cellular infrastructure of laticifers for the storage of latex. This hypothesis is based on the location of the main vascular bundles in a capsule, which trace a course through the mesocarp and terminate in the stigmatic rays (Kapoor 1995). Thus, the number of stigmatic rays is indicative of the major traces of laticifers in the capsule wall.

The linear nature and the strength of the relationship between capsule size (g) and latex mass (Table 1.1; $r = 0.89$, $P = 0.0001$) demonstrates a concomitant increase in these traits, which suggests that the latex mass is fixed per unit mass of capsule tissue. This relationship held across seven sites, which encompassed two geographic regions and where sites differed in soil type, microclimate and agronomic practices. Further, the sampled capsule material covered a range of site morphine yields and mean capsule sizes, lending strength to the observed consistency and linearity in this relationship.

Additionally, when latex mass was expressed per unit tissue (latex capacity), it was apparent that values were very similar across the sites at each stage (Figure 2.2a). This consistency reflects the strong genetic influence on latex yield (Jain *et al.* 2005, Singh *et*

al. 2000) and also demonstrates the importance of removing the effect of capsule size when investigating capsule relationships.

It was also apparent that latex capacity was fixed relatively early in development, and this was evident in the timing of latex accumulation. Latex capacity (mg latex/mg DW capsule) had generally already reached a maximum at sample stage 1, one week after flowering (Figure 2.2a). Bernáth (1989) demonstrated that one third of a capsule's dry mass had already developed by flowering stage, and it would appear that the laticifer system is also well developed by this stage (i.e. the proportion of tissue given to laticifers is in place).

At sample stage 1, the majority of experimental sites had peaked in their latex capacity i.e., the proportion of capsule wall tissue given to laticifers was set. This challenges the standard descriptions of an increase in laticifer development up to two weeks after the time of flowering. Fairbairn and Kapoor (1960) found that laticifers were most abundant in the outer mesocarp at two weeks after flowering, and Kapoor (1995) suggests that these laticifers (from the valve traces) contribute most to the yield of latex. The results of this study, however, suggest that any further development in laticifers would only be as a fixed proportion of the capsule wall, as further significant development in the system of laticifers would have resulted in latex capacity being greater at sample stage 2 than sample stage 1. To elucidate the exact timing and development of the system of laticifers in poppy capsules, new imaging technologies such as Nuclear Magnetic Resonance (NMR) could be employed (Glidewell *et al.* 1999, Goodman *et al.* 1992, Ishida *et al.* 2000). NMR techniques are now capable of investigating plant cells with resolution at the micron level (Ciobanu and Pennington

2002) and, coupled with the ability to non-intrusively investigate plant cells, it would be an ideal method to further examine the suggestion that latex capacity is fixed early in poppy capsule development.

The significance of capsule latex capacity to the end-yield of morphine was also investigated. Figure 2.2a demonstrated that latex accumulation occurred early in capsule development (greatest at one week after flowering), but the accumulation of morphine exhibited a different pattern (Figure 2.2b). Morphine continued to accumulate in the capsule beyond sample stage 2 until the time of final harvest, and this is consistent with other studies on morphine accumulation in poppies (Bernáth 1989). It is notable that the mass of morphine in the capsule continued to increase beyond the point when the laticifer system reached capacity (Table 1.1 and Figure 2.2b). This implies that the storage capacity of laticifers is not important in terms of morphine yield as most of the accumulation of alkaloid occurs after the amassing of latex (and development of the laticifers).

Additionally, the bivariate analysis using ASReml showed no correlation between the latex capacity of a capsule at either sample stage 1 or 2 and the final yield of morphine (at the time of commercial harvest), which indicates that the morphine end-yield was not predicated upon capsule latex capacity at stages 1 or 2. In fact, the best morphological predictor of final morphine yield was the dry capsule mass, which supports previous studies of alkaloid yield in poppies.

Although the results suggest that latex capacity is not a key yield component within cultivars, this study has not addressed the possible differences that may occur between

cultivars in terms of latex capacity and alkaloid yield, nor has it investigated the structural components of latex (vesicles) which may have an impact on yield. Morphine continues to accumulate after the laticifer system is set, and as the storage of this alkaloid is ultimately within the latex vesicles, it may be that these structures within the latex are more important to yield than the latex capacity of the capsule. Nessler and Mahlberg (1977a, 1979i, 1979ii) and Bock *et al.* (2002) have described the development and capping of vesicles within laticifers, and it has been demonstrated that the pellet component of latex increases after flowering (Pham and Roberts 1991). It is unclear, however, whether the pellet increase is a result of the vesicles increasing in size or in number, and whether they are concentrating greater quantities of alkaloid either from synthesis in the capsule or through translocation to the capsule. These detailed questions were beyond the scope of this study, but from the investigation undertaken, we can challenge previous descriptions of an increase in laticifer development up to two weeks after the time of flowering, and assert that the latex capacity of a capsule is not a major determinant in capsule yield.

2.6 Conclusion

In this study, the latex capacity of field grown poppy capsules was determined using a novel calculation approach. It was observed that the latex capacity (mg latex/mg DW capsule) is fixed early in capsule development; prior to the stage of one week after flowering. This challenges the descriptions of an increase in laticifer development up to two weeks after the time of flowering. Additionally, there was no detectable correlation between the latex capacity of a capsule and the final morphine yield of a capsule, and it was demonstrated that the most important characteristic in terms of alkaloid yield (morphine) at final sample stage was capsule size.

CHAPTER 3 Reproductive biomass allocation, plant architecture and yield components in field crops of *Papaver somniferum* L.

3.1 Abstract

The within-plant allocation of biomass to reproductive structures was assessed in field crops of the opium poppy, *Papaver somniferum* L. In excess of 80% of the total capsule mass accumulated on a plant was allocated to capsules at positions 1, 2 and 3. Seed mass yields were strongly and positively correlated to capsule husk yields. There was no significant difference between study sites in the per-plant productivity of capsule mass, and bud senescence was prevalent on the lower branches. The findings indicated that in the development of this commercial line of poppies there was sequential regulation of the sinks (fruit), and that the majority of reproductive biomass was allocated to the early developing capsules in terms of both fruit and seed. An argument is presented to suggest that developmental processes and resource limitation influence the allocation of biomass to reproductive structures in poppy, regulated by intrinsic plant mechanisms that ensure the full development and maturation of reproductive structures. Further studies are recommended.

3.2 Introduction

Patterns of biomass allocation in the opium poppy *Papaver somniferum* L. are of great interest, especially the allocation of dry matter to the reproductive structures, as capsules have the dual commercial importance of containing alkaloids in the husk material, and they contain seeds. Biomass investigations in other species (e.g. tomato, wheat) have assisted in the understanding of plant functioning and reproductive success, and for crop species, it has resulted in tangible outcomes by providing valuable information used for the optimisation of crop yields (Heuvelink and Buiskool 1995, Marcelis 1994, Salazar *et al.* 2008, Vieira *et al.* 2009). In poppies, it has been demonstrated that capsule mass is one of the strongest predictors for alkaloid yield (Harvest *et al.* 2009, Ozturk and Gunlu 2008, Singh *et al.* 2003), so the investigation of biomass allocation to reproductive structures may provide insights useful in commercial poppy crop management.

The accepted fundamental principle underpinning biomass allocation is that the distribution of resources to structures is dependent on resource availability, and that partitioning is driven by sink strength (Heuvelink 1996, Marcelis 1994, Salazar *et al.* 2008, Valantin *et al.* 1998). Indeed, the majority of plant growth models have incorporated a strong competitive component in the allocation of biomass within plants, and often the results of investigations are interpreted in a competitive framework (de Reffye *et al.* 2008). Many authors adhere to the concept of within-plant competition, and have attributed the growth of root and shoot structures, fruit and seed, to the effect of competitive interactions between sources and sinks (Susko and Lovett-Doust 1999). Competition has often been cited as the explanation for the development of fruit size

often at the expense of vegetative and generative structures (González-Real *et al.* 2008, Stephenson 1981, Valantin *et al.* 1998), and for decreased fruit size with increased fruit number on a plant (Yadegari and Barzegar 2008).

Other research, however, has proposed models with a greater conceptual complexity of allocation beyond that of competition mechanisms, incorporating developmental, physiological and genetic components. Observed trends in biomass allocation (such as reduced flower size, fruit size and lower seed set across a season) have been described in terms of integrated processes; where a combination of feedback mechanisms, sequential development, competition, and functional and genetic constraints contribute to patterns of allocation (Buide 2008, Wolfe and Denton 2001, Sadras and Ford Denison 2009). This has also been recognised in allocation to poppy capsules, with Bhandari *et al.* (1989) and Ghiorghiță *et al.* (1990) both acknowledging the role of development in capsule yield (although neither clarified this).

3.2.1 Poppy morphology and development

P. somniferum is an annual crop growing from 90 cm to 120 cm in height, usually with several lateral branches arising from the main stem. Leaves are sessile and function for a finite duration in poppies; the older leaves senesce as the plant develops. A single flower is produced on the apex of the main stem and at the terminus of each lateral branch (Petri and Mihalik 1998).

Both the branches and reproductive organs are produced successively, and the development of the vegetative and floral parts overlaps (Ghiorghiță *et al.* 1990). The flower on the main stem opens first and thereafter the order is basipetal (Petri and

Mihalik 1998). The flower size declines on successively lower branches, and the fruit (capsules) reach maturation sequentially. *P. somniferum* capsules are commonly globose with a flat stigmatic disc, and cultivars are non-dehiscent so seeds remain in the capsule (Kapoor 1995). Morphine is located predominantly in the capsules of the plant, with morphine yield reported as greatest in quantity in the capsule of the main stem and successively lower in lower order capsules (Shukla *et al.* 2004, Khanna and Gupta 1982, Loftus Hills 1945).

3.2.2 Growth relationships in poppies

Several studies have investigated the growth relationships in *P. somniferum*. Within populations, the effect of inter-plant competition through increased planting density has been recognised as retarding height, flowering, fruit size, the number of capsules per plant, seed weight per plant and in delaying maturity (Bhandari *et al.* 1989, Sip and Skorpik 1980). Further to this Laughlin (1987) and Chung (1990) found that capsule morphine yield was also compromised, but Bhandari *et al.* (1989) did not record an impact of planting density on morphine yield.

Growth relationships have also been investigated within the plant. Mika (1955) found an increasing, proportional relationship between the mean number of nodes and the mean dry weight of capsules. Positive correlations have also been reported between the number of capsules per plant and plant height; the main capsule width and plant mass; and between opium and seed yields and the number of capsules per plant, the capsule size and mass, and stem diameter (Singh *et al.* 2003, Shukla and Khanna 1987, Kaicker *et al.* 1978). Not all authors concur with these relationships, however, with both Muchova *et al.* (1993) and Tiwari *et al.* (2000), citing negative associations between the

number of capsules per plant and seed yield. Further, these previous investigations have ascertained associations at the broad plant level, and have not elucidated the within-plant processes of allocation and partitioning.

It is unclear whether the patterns of reproductive biomass allocation within the poppy plant are static or whether they are altered by the plant architecture. Research has not adequately addressed whether there is a differential pattern of allocation to reproductive structures according to their position on the plant, or if there is an impact of the degree of branching on allocation. This relationship is very important because the upper capsules are the major source of alkaloid yield on the plant, so understanding within-plant partitioning is key to maximising allocation to these structures, thus enabling yield improvements.

This study investigated the within-plant patterns of allocation to reproductive structures in commercially grown field crops. Commercial crops are planted with known genotypes; this limits genetic effects on results and also provides direct application for yield improvement strategies. The aims of this investigation were:

1. to assess the biomass allocation in field grown poppy plants to determine the patterns of within-plant partitioning to reproductive structures
2. to relate these patterns in biomass allocation to yield components, and
3. to ascertain those traits that are linked to greater capsule mass in the upper capsules of *P. somniferum*.

3.3 Materials and Methods

3.3.1 Sample collection and measurement

During the Tasmanian summer month of January 2006, *P. somniferum* plants at dry maturity stage were collected from four commercial field sites in southern Tasmania. At least 20 plants were removed from each of four random blocks within the field sites. Thus, each site was represented by a minimum of 80 poppy plants, with a total harvest from all four sites of 345 plants and 1017 capsules.

Plant density was calculated at each removal position (prior to plant removal) by counting the number of plants in 1 m². For each site, productivity per plant was calculated by dividing the total capsule mass of the block by the number of plants collected in that block. The productivity per unit area was calculated by multiplying plant productivity by density.

The removed plants were bagged and later measured for height, the number of nodes, the number of branches, apical stem diameter (ASD, stem diameter underneath the main capsule), basal stem diameter (BSD, stem diameter at the soil level), and stem mass. Capsule traits were also recorded, including; capsule height, diameter, number of stigmatic rays, and the position on the plant. The capsule height was measured from the base of the thalamus to the top of the stigmatic plate and capsule diameter was recorded as the mean of two measurements taken at the widest part of the capsule; perpendicular to each other. Capsule height and all recorded diameters were measured using electronic vernier callipers.

Capsule position was described as; capsule 1 for the capsule on the main stem, capsule 2 for the first lateral capsule, capsule 3 for the next lateral down etc. (Figure 3.1).

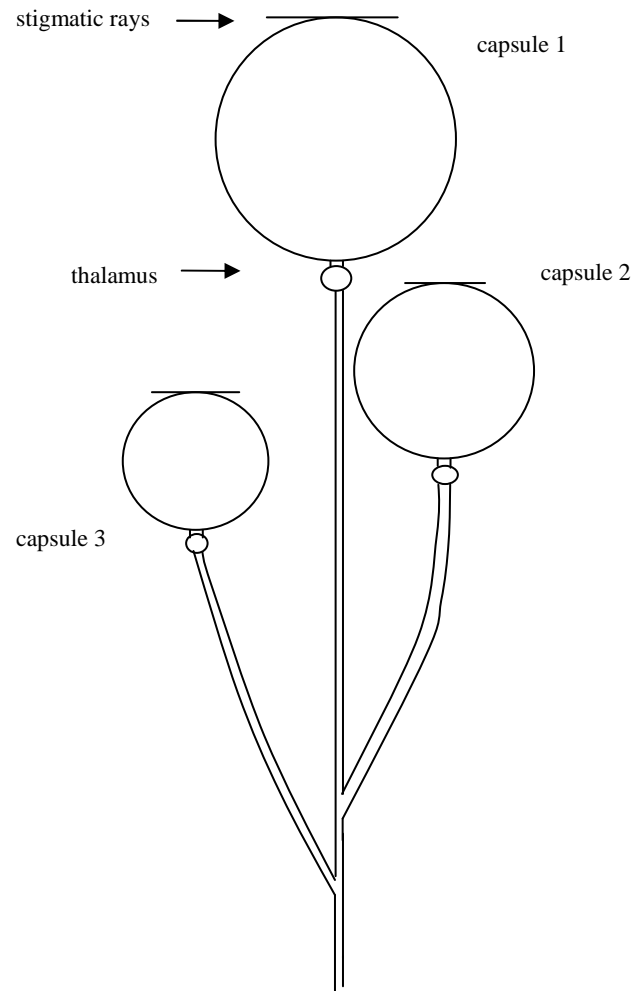


Figure 3.1. Branching schematic of a *P. somniferum* plant where capsule 1 is located at the apex of the main stem, capsule 2 on the first lateral, capsule 3 on the second lateral.

Also recorded for every plant was the capsule presence (1) or absence (0) at each branch tip. On the branch tips of a proportion of plants there remained a senesced, unopened flower bud and fruit was not set – this was denoted '0'. Finally, a small number of capsules were recorded as infertile. These capsules (denoted as present, '1') produced no seeds, instead containing small amounts of light brown, unfertilised ovule tissue.

3.3.2 Subset treatment

A subset of plants was chosen, screened on the basis of architecture. Plants representing the most common architecture were selected; those with a main stem axis and two lateral branches, all terminating in a capsule. This produced a sample size of 10 plants from each site; a subset totalling 40 plants and 120 capsules. All capsules were processed individually by first removing seeds and grinding the capsule husk (wall material) to a particle size of < 1 mm using a Glen Creston hammer mill. The ground capsule husk was solvent-extracted, the extraction slurry was centrifuged at 10,000 rpm for two minutes and the supernatant was assayed using high performance liquid chromatography (HPLC): Waters Alliance HPLC (Alltech Platinum C18, 3 μ m, Rocket Column; wavelength 254 nm; column temperature 40° C; pump speed 1.5 mL min⁻¹; injection volume 15.0 μ L; and water:acetonitrile gradient 2% to 32% acetonitrile over 18 min). Samples were assayed for morphine content only (there is a strong relationship between total alkaloid content and the morphine content in capsules, Dittbrenner *et al.* 2009).

3.3.3 Data analysis

3.3.3.1 Productivity per unit area and productivity per plant

The differences between sites in productivity per unit area (capsule mass (g)/m²) and in productivity per plant (capsule mass (g)/plant) were both assessed using a one-way ANOVA in Minitab 11.12. The data for productivity per unit area were not normal and variances were heterogeneous, so data were log transformed to meet the assumption of the test. Tukey HSD was employed for post hoc analysis.

Linear regressions were applied to assess the relationship between productivity per unit area (log transformed data) and density, and productivity per plant and density.

3.3.3.2 Plant architecture at each of the four field sites

The association between site and plant capsule number was assessed using a Chi Square analysis of the frequencies recorded for the categories of number of capsules per plant. The frequencies for plants with six or more capsules were grouped into a category of '≥6' for each site, as there were very few plants with such a high degree of branching.

A Spearman Rank correlation was conducted to ascertain the relationship between density, the number of branches on a plant, and the number of capsules on a plant in SPSS Statistics 17.0.

3.3.3.3 *Within plant biomass allocation to capsule husk and seed*

To determine the predictive ability of morphological variables on the size of reproductive structures (the mass of the capsule husk and the seed mass), a stepwise linear regression was conducted on the data using the statistics package, SPSS Statistics 17.0. Capsule husk mass and seed mass were assessed separately as each has individual commercial importance.

As one of the intentions of this investigation was to elucidate the influence of architecture on the allocation to particular capsule positions, each position was assessed individually. Note that for all regression models applied to the data, including the logistic regression below, models were limited to capsule position < 6, as beyond this there were insufficient data to support relationships. Data were assessed for normality and homogeneity of variances. Stem mass was cube root transformed to meet the regression assumptions. Confidence intervals (CI) were presented for the regressions, as they were deemed more appropriate than the standard error (Streiner 1996).

The presence/absence data scored for the capsules were analysed using a logistic regression for binary data, conducted in R 2.9.2. As each capsule position was not considered independent of the others within a plant, the regression model included both capsule position and capsule number. The probability of lack of fruit set (capsule absence, '0') at a given capsule position, accounting for the number of capsules on the plant, was assessed using the following model, viz:

$$glm(formula = Absent \sim factor(Capsules) + Cap_position, family = binomial, data = d5)$$

Finally, a general linear model (GLM) was constructed to assess the significance of capsule position on morphine yield (capsule concentration) for subset data. Capsule concentration of morphine was modeled rather than capsule morphine mass in order to remove the effect of capsule size. Tukey HSD was employed for post hoc analysis. The model was developed in the statistics package, SPSS Statistics 17.0.

3.4 Results

3.4.1 Description of field crops; productivity and plant morphology

The four Tasmanian sites that were studied differed in both productivity and plant morphology. The one-way ANOVA identified a significant difference between sites in productivity per unit area ($F_{3,12} = 6.74$, $P = 0.006$), with the productivity per unit area at Site 1, although variable, much greater than that of the three other sites (Table 3.1). A post hoc analysis demonstrated that Sites 2, 3 and 4 were not significantly different from one another in productivity per unit area.

Table 3.1. Summary of measured plant parameters for sites in southern Tasmania. ASD; the apical stem diameter measured underneath the main capsule and BSD; the mean stem diameter measured at the base of the plant. Productivity per unit area = total capsule mass m^{-2} and productivity per plant = total capsule mass / no. plants. \pm SEM shown for mean values.

Site	1	2	3	4
Productivity per m^2 (g)	639.4 \pm 75.8	384.9 \pm 33.2	404.5 \pm 54.7	331.2 \pm 19.3
Productivity per plant (g)	13.02 \pm 0.81	12.41 \pm 2.29	13.92 \pm 0.53	9.91 \pm 1.31
Mean site density (plants/ m^2)	49.17 \pm 4.98	33.44 \pm 5.30	28.94 \pm 3.57	34.67 \pm 3.65
No. plants harvested	82	83	94	86
No. capsules harvested	267	219	277	254
Maximum capsule number	6	6	6	7
Maximum branch number	7	6	9	8
Plant height (cm)	109.13 \pm 0.99	107.30 \pm 1.38	115.87 \pm 1.00	99.95 \pm 1.51
ASD mean (mm)	5.48 \pm 0.06	5.35 \pm 0.06	5.54 \pm 0.05	5.11 \pm 0.07
BSD mean (mm)	11.63 \pm 0.29	10.56 \pm 0.25	12.45 \pm 0.34	13.04 \pm 0.40
Mean stem mass (g)	12.09 \pm 0.64	13.20 \pm 1.08	13.36 \pm 0.71	10.50 \pm 0.63
No. of nodes/plant	19.50 \pm 0.48	16.36 \pm 0.40	17.35 \pm 0.37	17.99 \pm 0.50

The four sites varied in planting density and a linear regression showed that density had a significant, if very weak, positive effect on productivity per unit area ($y = 2.31.89 + 0.009 \text{ density}$; $P = 0.002$, $r^2 = 49.6$). The one-way ANOVA for productivity per plant however, revealed no significant difference between sites in the total capsule mass per plant ($F_{3,12} = 1.49$, $P = 0.266$), and the linear regression revealed that there was no effect

of density on the total capsule mass per plant ($y = 15.51 - 0.09 \text{ density}$; $P = 0.211$, $r^2 = 10.9$). There was a significant but weak correlation between density and the number of branches on a plant but only at Site 2 ($\rho = -0.26$, $P = 0.019$) and Site 3 ($\rho = 0.25$, $P = 0.016$). There was also a significant but weak correlation between density and the number of capsules on a plant at Site 2 ($\rho = -0.42$, $P = 0.001$) and Site 3 ($\rho = 0.21$, $P = 0.044$).

Sites differed in the morphology of constituent plants, with variation in plant height, stem diameters and stem mass (Table 3.1). It was also evident that the sites differed in the degree of plant branching and the disparity between maximum branch number and maximum capsule number showed that not all branches terminated in a capsule. The association between sites and number of capsules per plant was assessed using a Chi Square analysis of the frequencies of plant types (Table 3.2).

Table 3.2. Chi Square contingency table presenting the frequency of plants bearing a certain number of capsules at each of four field sites, southern Tasmania. Observed frequencies are presented in the top line of each plant type, expected frequencies are presented below.

No. capsules	Site 1	Site 2	Site 3	Site 4	Total
1	7 7.61	7 7.7	6 8.72	12 7.98	32
2	14 22.58	30 22.86	30 25.88	21 23.68	95
3	31 29.47	37 29.83	29 33.79	27 30.91	124
4	17 14.74	6 14.92	22 16.89	17 15.46	62
5	8 4.52	1 4.57	6 5.18	4 4.74	19
≥6	5 3.09	2 3.13	1 3.54	5 3.24	13
Total plants:	82	83	94	86	345

There was an association between site and the frequency of plants bearing a certain number of capsules (χ^2 29.88, df = 15, P = 0.012). Site 1 contained fewer than expected numbers of plants with 2 capsules, but had greater than expected frequencies of plants with more numerous capsules (3, 4, 5, ≥ 6). Site 2 had a greater proportion of plants bearing 2 and 3 capsules, but fewer than expected plants with 4, 5 and ≥ 6 capsules. Site 3 also contained numerous plants bearing 2 and 4 capsules, but Site 4 contained notably more single-capsule plants than expected, and this was the site that recorded the lowest productivity per plant (Table 3.1).

Although the studied sites were significantly different in their frequency of plant types, all sites closely followed the same pattern of the proportion of capsules represented at particular positions (Figure 3.1).

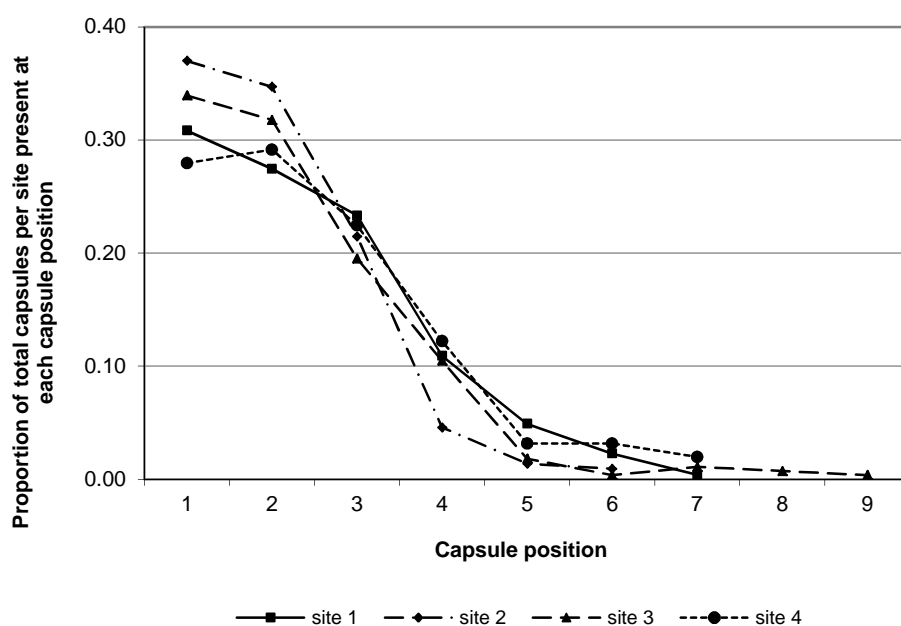


Figure 3.1. The number of *P. somniferum* capsules at a given plant position presented as a proportion of the total number of capsules collected for that site. Data are presented for each site.

For all sites, the capsules at the upper 3 positions comprised greater than 80% of the total number of capsules collected at the site, and for all but site 4, the main capsule (position 1) was most numerous. The capsule at position 1 also had the highest mean capsule husk mass for most plant architectures and across all sites (Figure 3.2).

3.4.2 Distribution of capsule husk mass (net mass, no seed)

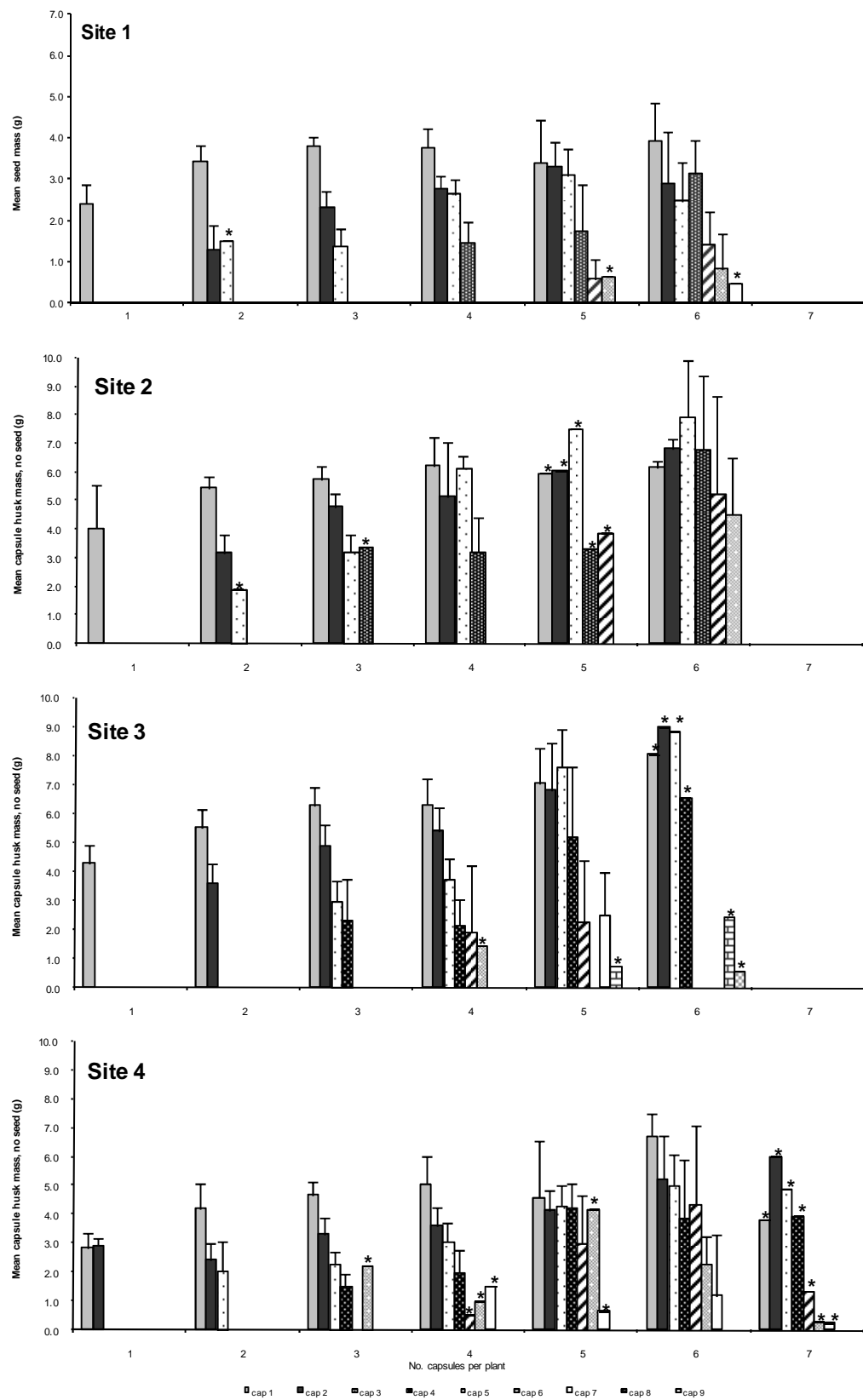


Figure 3.2. The mean capsule husk mass (g) of capsules by position on *P. somniferum* plants. The number of capsules per plant ranged from 1 to 7 and up to nine positions were represented. 95% confidence intervals are presented; * indicates $n = 1$.

It was found that mean capsule masses were greatest at Site 3 (which had the highest productivity per plant) and least at Site 4 (lowest productivity per plant, Table 3.1). It is also of note that mean capsule mass was either maintained or increased slightly at each capsule position as plants became more branched, until plants had 5 capsules. Beyond this the data were less reliable (evident in the confidence intervals).

As it was apparent that larger plants carried greater capsule biomass, the relative allocation of dry matter was examined. This revealed that of the total capsule husk mass on a plant of a given architecture, the proportion of capsule mass allocated to capsule 1 and capsule 2 decreased as plants developed more lateral branches (Figure 3.4).

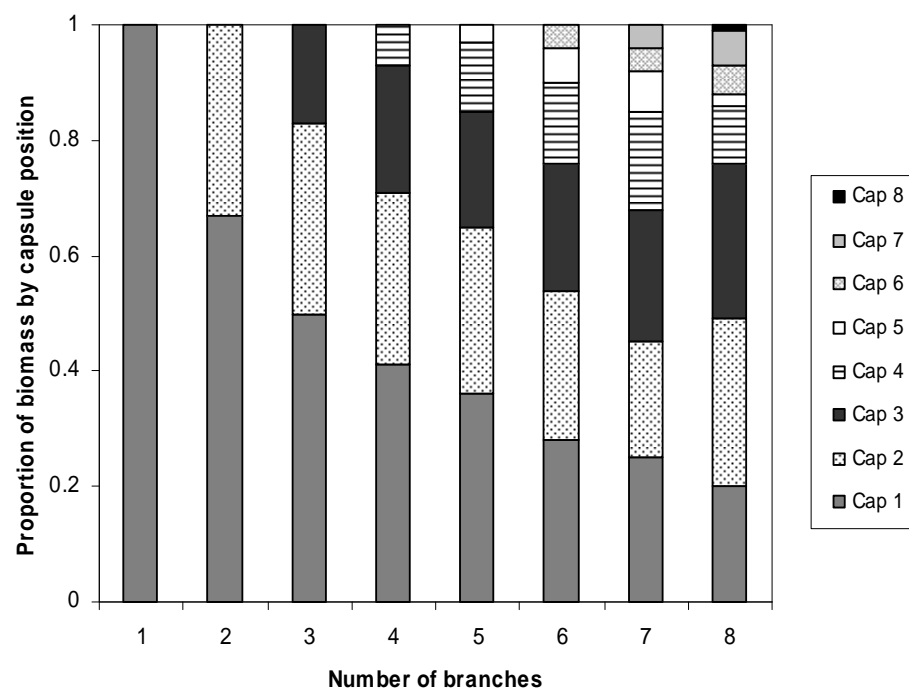


Figure 3.3. The proportion of mass allocated to capsules at each position, for every branching architecture of *P. somniferum* plants. The proportion was calculated by dividing the sum of the capsule mass for a given position by the total capsule mass on the plant, for plants of a given architecture. Thus, proportions were individually calculated for each of the eight branching architectures.

Note that although there was a shift in allocation to lower order capsules as branches became more numerous, it remained that the upper three capsules comprised the majority of the total capsule husk yield.

To provide a deeper insight into the allocation of capsule husk mass that was observed in Figure 3.2 and Figure 3.3, a regression was performed to investigate the morphological predictors of capsule net mass (accounting for position). Stem mass (transformed) was found to be a positive predictor at all positions and was the major contributor in explaining the capsule net mass variable (Table 3.3).

Table 3.3. Stepwise linear regression models for five capsule positions on sampled *P. somniferum* plants. Descriptors that were retained: caps, number of capsules on a plant; hgt, plant height; SM, stem mass (cube root transformed).

	Model	R ²	adj.	Sig.
Cap	$y = 1.28SM - 0.23caps + 0.01hgt - 1.20$	0.62	0.62	0.0001
Cap	$y = 1.83SM - 0.19caps - 2.06$	0.	0.	0.0001
Cap	$y = 1.72SM - 2.74$	0.70	0.70	0.0001
Cap	$y = 1.90SM - 0.02hgt - 2.08$	0.64	0.63	0.0001
Cap	$y = 1.92SM - 0.02 hgt - 2.02$	0.55	0.51	0.0001

Also of note is that for both capsule positions 1 and 2, the predictors of capsule husk mass included the number of capsules on a plant, a parameter that had a negative effect on the capsule net mass at these positions.

3.4.3 Distribution of capsule seed mass

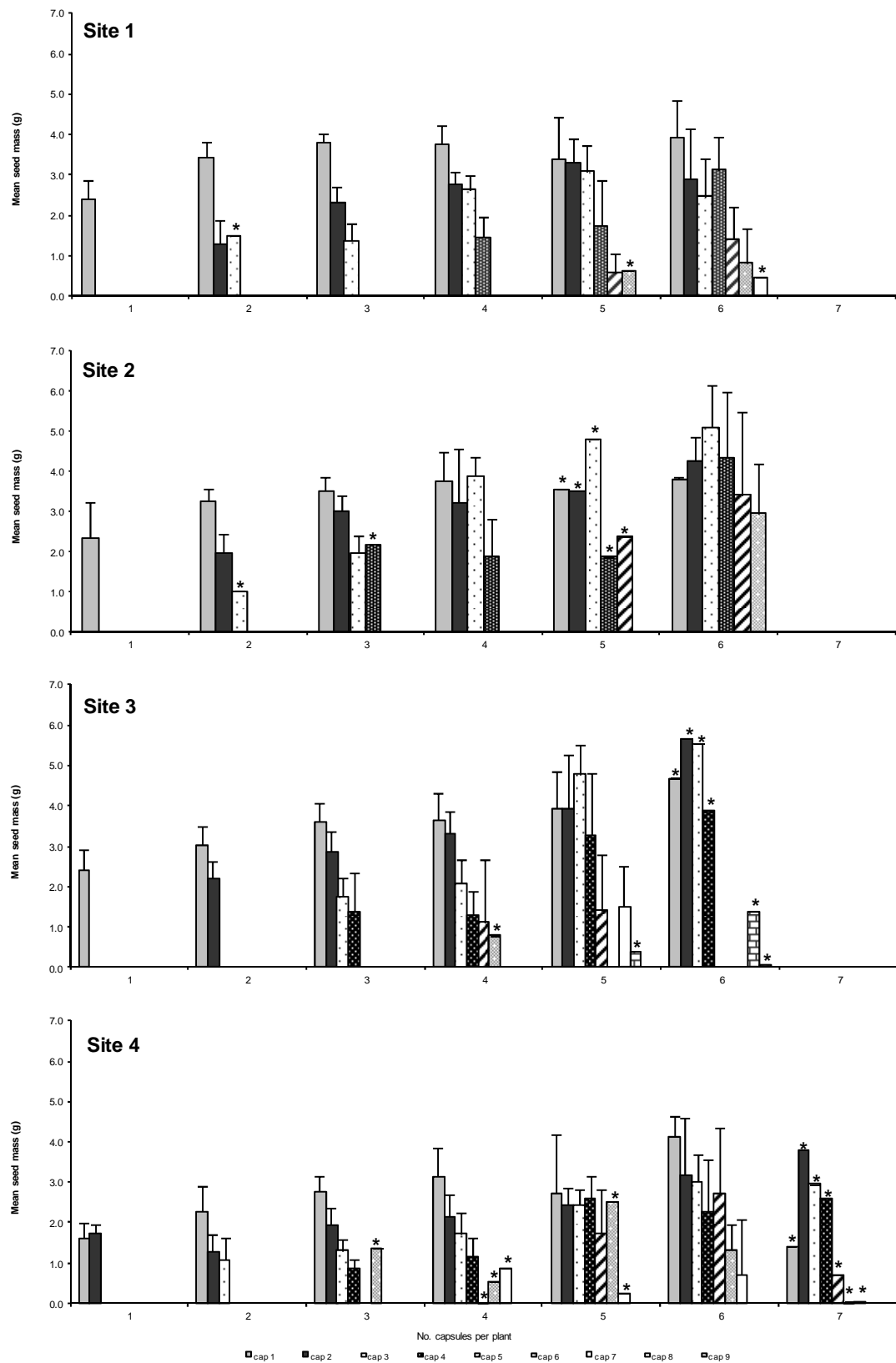


Figure 3.4. The mean capsule seed mass (g) of capsules by position on *P. somniferum* plants. The number of capsules per plant ranged from 1 to 7 and up to nine positions were represented. 95% confidence intervals are presented; * indicates $n = 1$.

The pattern in allocation to seed mass was consistent with that observed for the mean capsule husk mass at each position, and the proportional allocation of dry matter to seed biomass was also congruent with that observed for capsule husk mass, in that the proportion of seed allocated to upper capsules was reduced to accommodate those capsules on lower order branches (Figure 3.5).

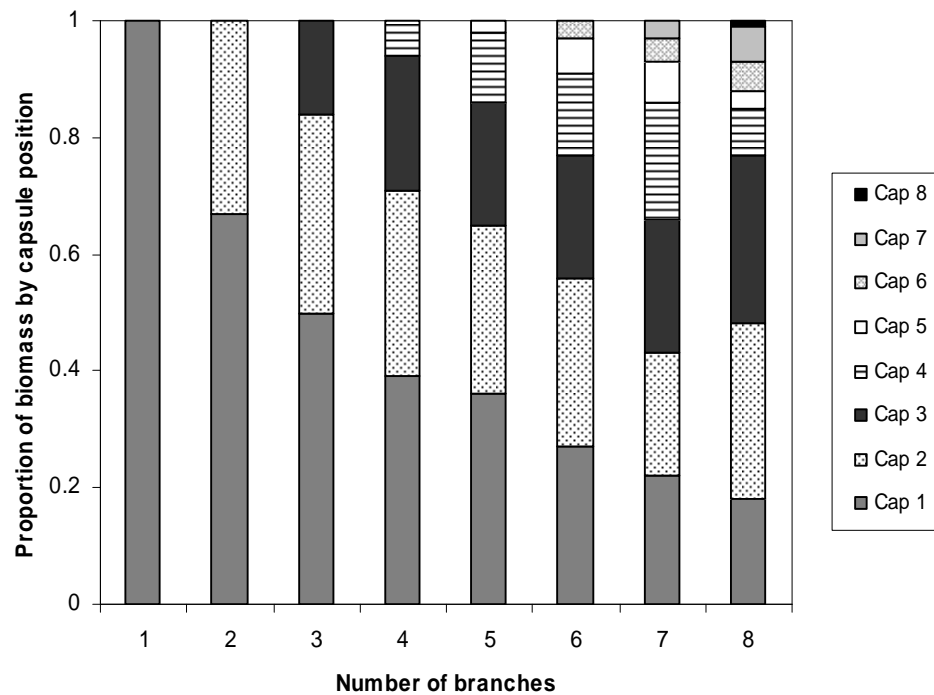


Figure 3.5. The proportion of seed mass contained in capsules at each position, for every branching architecture of *P. somniferum* plants. The proportion was calculated by dividing the sum of the seed mass for a given position by the total seed mass on the plant, for plants of a given architecture. Thus, proportions were individually calculated for each of the eight branching architectures.

The visible agreement between the trends for capsule husk and seeds in terms of average mass and relative proportions was supported by the strong positive correlation between the mass of capsule husk and the mass of seed contained in that capsule ($r_s = 0.855$, $P < 0.01$).

Further to this, there was a strong upper limit to resource partitioning within a capsule, where the seed mass within a capsule comprised no more than 70% of the total mass of that capsule (Figure 3.6).

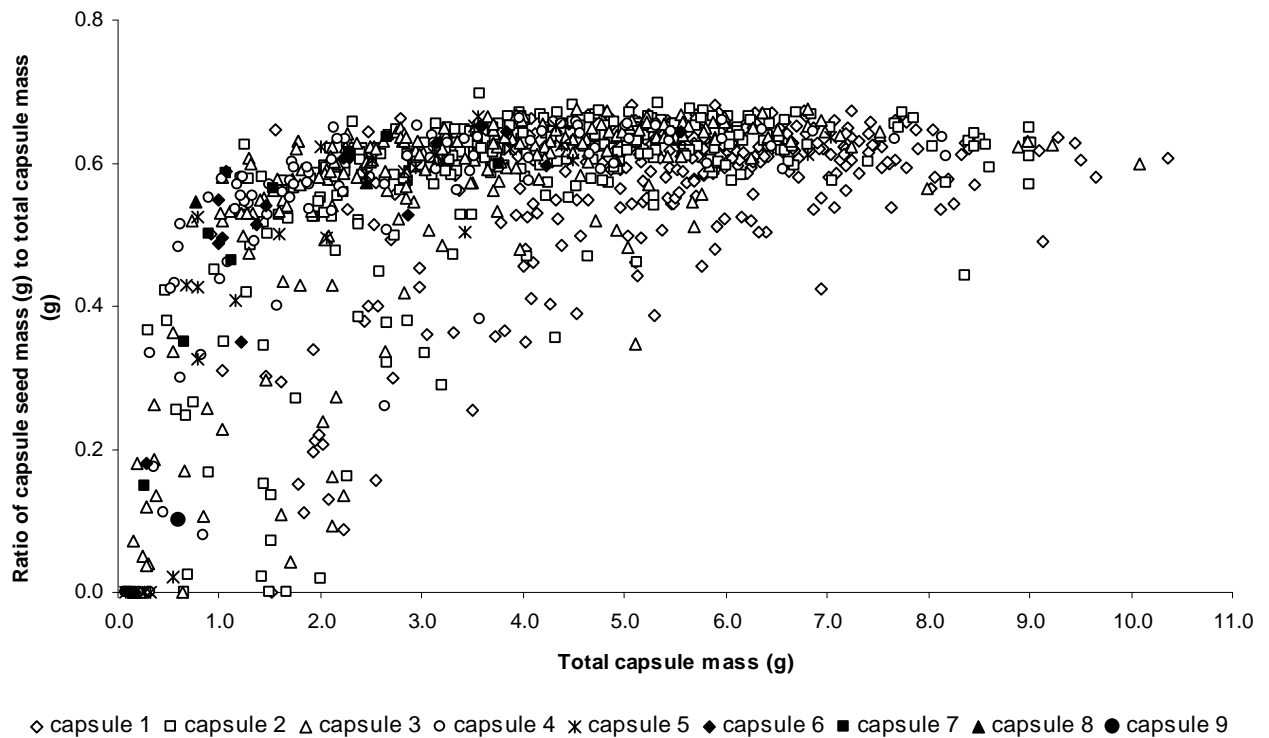


Figure 3.6. The ratio of seed mass (g) to total capsule mass (g) for *P. somniferum* capsules, plotted against total capsule mass (seed + husk) (g). Individual data points represent a capsule, $n = 1017$.

The upper limit of partitioning to seed mass was evident for all capsule positions. Additionally, capsules at all positions consistently contained a high ratio of seed mass for the majority of total capsule mass values, but where total capsule mass was less than 1 gram the ratio of seed mass was variable.

Infertile capsules represented 2% of the total number of capsules sampled ($n = 1017$) and were present at all sites (Figure 3.6, represented on the x axis). They were most

frequent at capsule positions 3, 4 and 5 and on average each had a low total capsule mass (mean $0.42\text{g} \pm 0.11$ SEM).

3.4.4 Fruit production

It was evident during sampling that not all flowers opened and set fruit; in place there was a senesced, unopened flower bud. This occurred on 151 of the 345 plants sampled. Bud senescence was more prevalent on plants that were more highly branched, and the proportion of plants that did not set fruit at one or more flowers linearly increased with branching, for each site (Figure 3.7). The final data point for Site 2 was the exception.

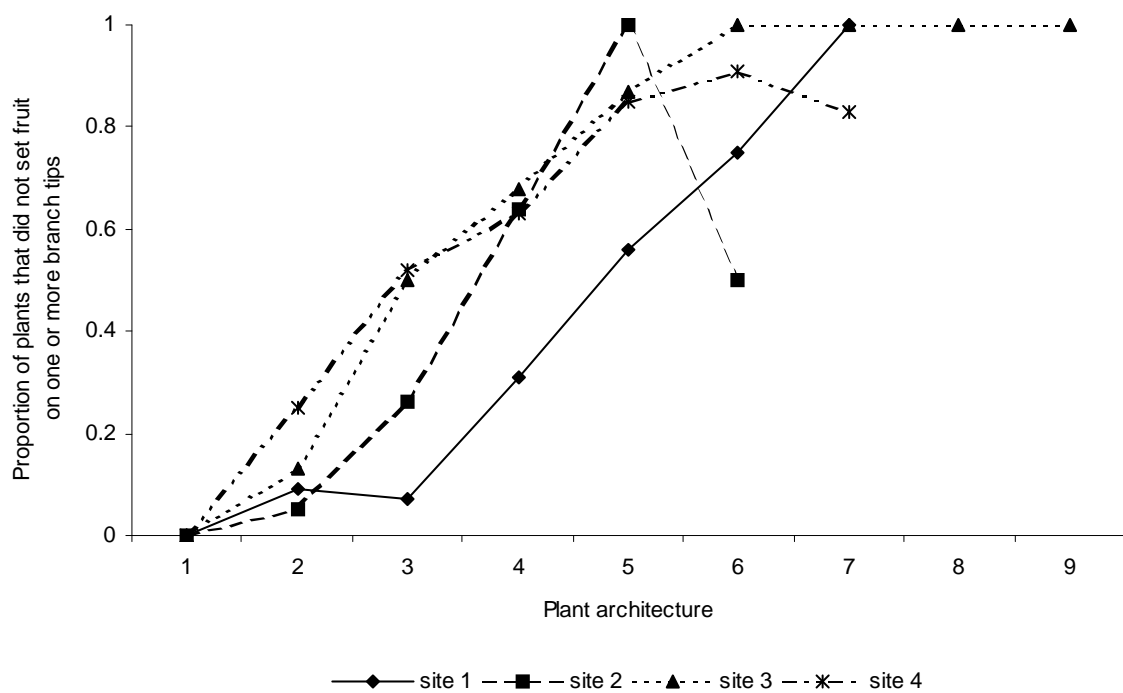


Figure 3.7. The proportion of plants that did not set fruit on one or more branch tips of *P.somniferum* plants. Plant architectures are represented for each of the four Tasmanian sites studied.

Lack of fruit set occurred most frequently at the lower capsule positions on a given plant (Figure 3.8).

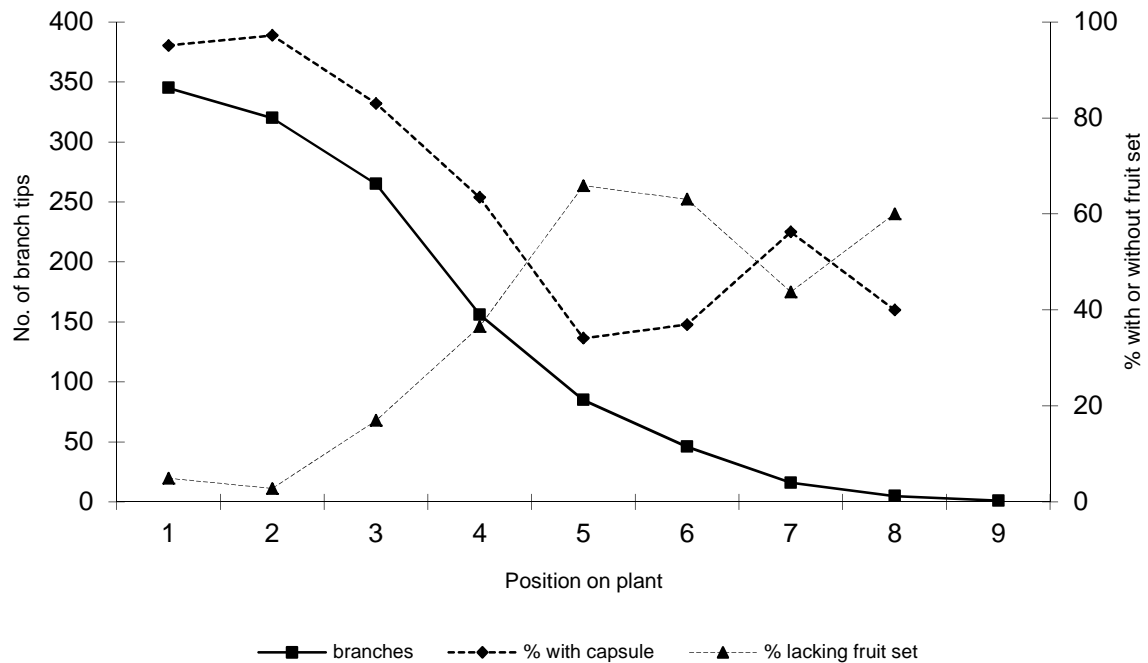


Figure 3.8. The production of a capsule and lack of fruit set (senesced, unopened flower) at branch tips on branched plants of *P.somniferum*.

For both positions 5 and 6, more than 60% of tips did not successfully set fruit. Beyond this position there were insufficient data to reliably support this trend. Note that the data point at position 9 was not illustrated in the figure, as there was only one representative plant.

While it was evident that capsule absence was more likely to occur on lower branches, in order to examine the effect of the presence of other capsules on the likelihood of bud senescence at a given branch position, data were analysed by logistic regression (Table 3.4). Capsule position was not considered independent of the others within a plant, thus the regression model addressed this effect (section 3.3.3.3).

Table 3.4. Logistic regression coefficients of capsule absence at different capsule positions on branched poppy plants. Null deviance: 1102.2 on 1149 degrees of freedom; residual deviance: 599.1 on 1144 degrees of freedom.

	Estimate	Std.	z value	Pr(> z)
(Intercept)	-4.0640	0.4838	-8.401	< 2e-16
factor(Capsules)2	-1.5486	0.5002	-3.096	0.00196
factor(Capsules)3	-3.7137	0.5505	-6.745	1.53e-11
factor(Capsules)4	-5.5454	0.6509	-8.519	< 2e-16
factor(Capsules)5	-7.4955	0.8178	-9.166	< 2e-16
Capsule position	2.0215	0.1416	14.278	< 2e-16

Significance codes: 0.0001 '***' 0.001 '**' 0.01 '*' 0.05 '.'

The regression revealed that within a plant, and accounting for the presence of capsules at other positions, plants with more capsules were more likely to record capsule absence at a particular position on the plant (Table 3.4). Also, the proportional odds ratio for capsule position (exponent of the estimate) was 7.6, demonstrating that for each unit movement in position the incidence of capsule absence (bud senescence) was 7.6 times more likely to occur. That is, bud senescence was much more likely to occur at sequentially lower capsule positions.

It is of note, however, that senescent flower buds were not always located at the lowest branch, and that for 25% of the plants that had senesced flower buds (37 of 151 plants), the successive branch produced a mature capsule.

3.4.5 Capsule position and morphine yield

A GLM was conducted on subset data for each site to assess the morphine concentration in capsules at each position. There was a significant position effect at site 2 ($P = 0.012$), at site 3 ($P = 0.001$), and at site 4 ($P = 0.004$). There was no significant difference

between the capsule morphine concentrations of the three positions at site 1 ($P = 0.625$). Generally the greatest concentration of morphine was present in the capsule at position 1 for three of the four sites, with subsequent capsules having lower concentrations (Figure 3.9).

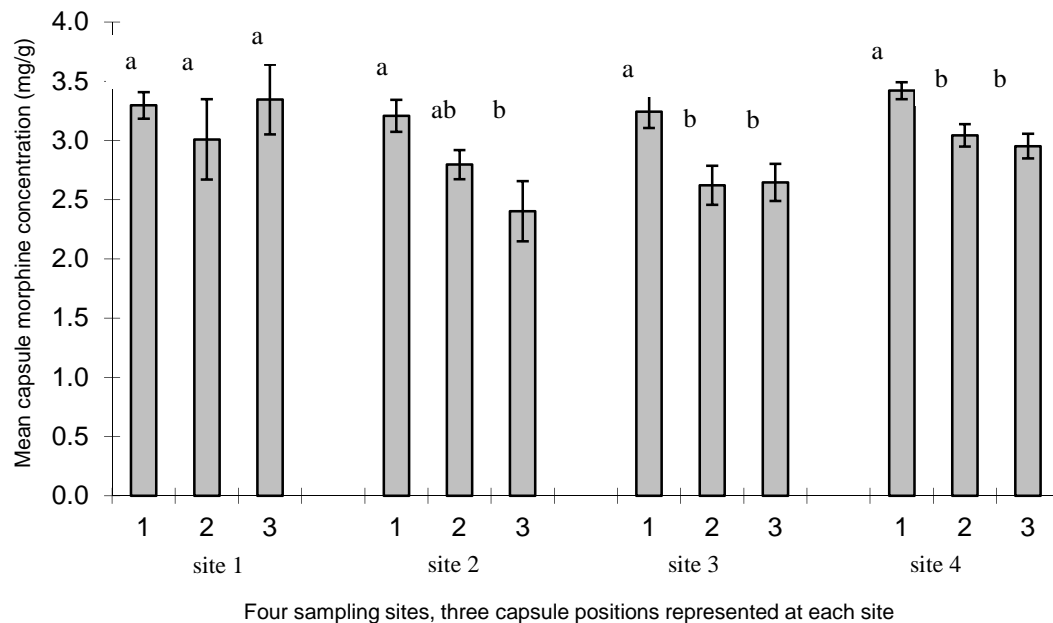


Figure 3.9. The mean morphine concentration in capsules for positions 1, 2 and 3. The four southern Tasmanian sites are presented. Error bars are shown, 1SEM, $n = 10$ at each site.

Post hoc analysis demonstrated that at site 2, only positions 1 and 3 were significantly different from each other ($P = 0.009$). At site 3, positions 1 and 2 were significantly different ($P = 0.002$), and positions 1 and 3 also differed significantly ($P = 0.002$). Site four displayed that same trend as site 3, where positions 1 and 2 ($P = 0.019$) and 1 and 3 ($P = 0.004$) were significantly different in capsule morphine concentration.

3.5 Discussion

3.5.1 Productivity per unit area and productivity per plant

Productivity per plant did not differ significantly between sites and the linear regression revealed that there was no effect of density on the total capsule mass per plant. Although previous studies have identified negative effects of density on growth and yields in *P. somniferum* (Bhandari *et al.* 1989, Sip and Skorpik 1980), in this study there was no effect of density on the capsule mass per plant.

These findings are consistent with the results of research by Chung (1990), in a Tasmanian study of *P. somniferum* population density. Chung (1990) investigated a range of planting densities (10-200 plants m⁻²) and found that there was little difference in plant characteristics below 50 plants m⁻², including total capsule yield. The author went on to recommend a planting density of 70 plants per m⁻² to maximise density effects. For the sites in this study, recorded densities were less than 50 plants m⁻² (Table 3.1) and therefore the lack of a density effect on plant productivity was predictable.

The previous work on planting densities also provides an explanation for the significant difference between Site 1 and the other three sites for productivity per unit area. Site 1 recorded the highest planting density and as there was no significant difference between sites in productivity per plant, more numerous plants m⁻² resulted in an increase in productivity per unit area. Based on earlier Tasmanian studies (Chung 1990, Chung 1987, Chung 1982), at the relatively conservative planting densities in these sites competition effects would be minimal. This may also explain the inconclusive Spearman Rank analysis of the relationship between density and plant morphology, and

thus it is suggested that further controlled studies are warranted to assess the influence of environmental characteristics.

Although there was no significant difference detected between sites in terms of the total capsule mass produced per plant, the frequency of plant types did vary significantly between sites, e.g. Site 1 had greater than expected numbers of plants that had 3, 4, and 5 capsules per plant, whereas Site 4 had a greater than expected frequency of plants with only one capsule (Table 3.2). The association between site and frequency of plant types did not result in site differences in plant productivity due to the numbers of representative types. At most sites the mode of capsules on a plant was three, and the contribution to total capsule mass from plants with more capsules or fewer capsules was relatively minor (e.g. only one plant of 83 at Site 2 had five capsules).

Additionally, the proportion of capsules contributing to the total capsules collected per site demonstrated the same trend at all sites, in that the majority of the capsules collected were from positions 1-3 (Figure 3.1), with capsules from the main stem generally the most numerous and usually the greatest in mass on the plant (Figure 3.2). Further, the mean mass of capsules at lower positions was usually less than that of the capsules position 1, 2 and 3 (Figure 3.2) and the mass of capsules at positions 1-3 comprised a much greater proportion of the capsule mass on a plant than those from lower positions (Figure 3.4). These trends were evident at all sites and explain the lack of a significant site difference in plant productivity, and they also have implications for the intrinsic regulation of biomass allocation to capsule positions within a plant.

3.5.2 Capsule net mass

The pattern of allocation of dry matter to capsule positions was similar for all sites, in that the capsule at position 1 usually had the greatest mean capsule husk mass, with successive capsules being sequentially smaller (Figure 3.2). This was also consistent across different plant types, although on the more highly branched plants (carrying ≥ 5 capsules) there was some variability.

Also, although there were increases in the mean capsule husk mass as lateral branches became more numerous, it was apparent that there was a limit to the relative capsule mass achieved at a particular position. The increases observed in mass (Figure 3.2) were most likely a result of a general increase in overall plant size, as indicated by the positive effect of stem mass on capsule husk mass (Table 3.3), but were not relative increases (Figure 3.3). The suggestion that capsules are limited to an effective size is also supported by the regression that demonstrated that the number of capsules on a plant negatively affected the capsule husk mass at positions 1 and 2 (Table 3.3).

The consistent, sequential patterns in the husk mass of capsules at a certain positions on a plant and the limit in proportional allocation of mass indicates that there are strong developmental and plant architectural influences on the distribution of biomass across capsule positions in *P. somniferum*. Similar mechanisms have been observed in other species (Buide 2008, Fourcard *et al.* 2008, Kang and Primack 1991, Wolfe and Denton 2001) and it has been hypothesised that allocation to reproductive structures during plant development is also mediated by fluctuations in maternal resource availability (Berjano *et al.* 2011, Lloyd 1980, Stephenson 1981).

The combination of developmental, architectural and resource factors does explain the weighted allocation to upper capsules in this field study of *P. somniferum*. The strength of a sink relates to the growth rate, developmental stage and the amount of seed set in the fruit (Valantin *et al.* 1999), and it may be suggested that in poppies, the capsule at position 1 has a developmental advantage as the apical flower of the main stem is produced first. Bussi eres (1993) discussed fruit sink strength in terms of metabolic activity and available surface area, and poppy capsules, which rapidly expand after fertilisation (Chung 1982), would operate as strong resource sinks. The result is that capsule 1 has both developmental and sink-strength advantages over subsequent capsules, and this is reflected in the consistency of mean capsule masses across sites (Figure 3.2). Beyond capsule 1 and in accordance with the sequential branch development that is characteristic in poppies (Petri and Mihalik 1998), in general the later capsules have successively less mass.

Limitation of resource availability and competition between the sinks during development were also evident. The most important morphological predictor of capsule net mass for all positions was stem mass (Table 3.3), which provides an indication of the positive influence of plant size on resource supply (Ba uelos and Obeso 2005). Similar effects of stem mass have been observed in other species, including the annual *Linaria canadensis* (Wolfe and Denton 2001). The significance of the number of capsules, also included in the regression model, may be posited as an indication of sink competition processes in *P. somniferum*. The number of capsules was demonstrated to negatively affect capsule net mass but only for capsule positions 1 and 2. The regression results for the capsule mass of later capsules (Table 3.3) identified that the significant predictors for net mass were stem mass and plant height, but not the number

of capsules. It is not surprising that there are different predictors for capsules on the same plant; for early capsules any successive capsule would potentially compete for resources while the early capsule was still developing (Bañuelos and Obeso 2005), but later capsules would not experience the same degree of competition as the sink strength of the earlier capsules would diminish as they reach maturation (Stephenson 1984). Thus, as indicated by the regression and consistent with poppy plant development (Chung 1982, Chung 1987, Petri and Mihalik 1998), the most important factor for the husk mass of a later-developing capsule was most likely the local supply of assimilates.

3.5.3 Capsule seed mass

Based on previous literature demonstrating the correlation between seed mass and capsule mass (Shukla and Khanna 1987, Singh *et al.* 2003) and observations that capsule to seed ratios remain similar under varying environmental treatments (Chung 1987, Chung 1990, Sip and Skorpik 1980) it is predictable that the patterns in biomass allocation to seed very closely followed that of the capsule husk (Figure 3.4, Figure 3.5). Further, considering the strong positive correlation with capsule net mass ($r_s = 0.855$, $P < 0.01$) and the consistent ratio of seed mass to total capsule mass (Figure 3.6), it is hypothesised that the factors predicting that variable will also predict seed mass (Shukla *et al.* 2003). As described in the accumulation of capsule net mass, the ability of the capsule to accumulate seed mass will relate to resource availability, and, as a stronger sink with a greater mass of developing seeds (in themselves a strong sink; Valantin *et al.* 1999), the early capsules have a developmental, competitive and resource supply advantage.

Other studies have identified competition and resource supply effects, and have stated that fewer capsules on a plant will result in greater seed mass per capsule (Muchova *et al.* 1993, Tiwari *et al.* 2000). The implications of Figure 3.6, however, suggest that gains from fewer capsules may be limited. In this field study, it appeared that the partitioning to seed mass within a capsule was tightly controlled, with the proportion of seed in a capsule consistent across capsule positions and such that the allocation to seeds did not usually exceed more than 70% of the total capsule mass. For most capsules the seed comprised approximately 60% of the total capsule mass, a figure that concurs with previous observations in poppies (Chung 1990). The data for small capsules (<1.3g) were scattered and inconsistent, but this was the case for capsules at all

positions as such and may be interpreted as an indication of the fitness of the capsule to support seed development.

The pollination of flowers does not appear to be of concern in field grown poppies; not only was the proportion of seed very consistent across capsule positions, there were very few infertile capsules (<2%) and these were represented across a range of positions. Although ineffective pollination of flowers has been cited as a factor in the limitation of reproductive output (Bañuelos and Obeso 2005), others have demonstrated that it is not a key factor in the limitation of fruit production (Berjano *et al.* 2011, Guitián *et al.* 2001, Stephenson 1984) and from this study it does not appear to be a factor for poppies. Finally, since seed mass and capsule mass were strongly and positively correlated in this study, they will be considered together in following discussion.

3.5.4 Control of fruit production

It is a biological imperative for initiated fruit that full development and maturation occurs (Stephenson 1981). Plants commonly regulate resource allocation by moderating competitive processes to ensure the supply of resources to developing fruit and seed, and there are several strategies to manage the allocation process. These include the abscission or abortion of fruit and flowers, or the senescence of floral structures, and they may be employed at several stages during the lifecycle (Bañuelos and Obeso 2005, Cawoy *et al.* 2007, Lloyd 1980). The first stage where regulation occurs is during the determination of flowers, and this appears to be the stage at which *P. somniferum* controls resource allocation within plants.

It is notable that the senescence of buds occurs prior to the production of a poppy capsule, before the flower opens. It was evident in this study that bud senescence was more prevalent in plants with a greater number of lateral branches (Figure 3.7) and that bud senescence occurred most frequently at lower capsule positions (Figure 3.8). Further, the likelihood of capsule absence was significantly more probable when there were greater numbers of capsules on a plant (Table 3.4). This, and the predominant localisation of bud senescence to lower branches is suggestive of developmental restraint through controlled or programmed organ loss, in order that sufficient resources are made available to support the full development of the uppermost capsules (Sadras and Ford Denison 2009). Programmed organ death has been previously described (Sadras and Ford Denison 2009, Stephenson 1981) and suggested triggers include assimilate limitation and hormonal controls. It is probable that assimilate limitation may be a factor in poppy bud senescence on lower branches as these continue to develop when leaves on the main stem are beginning to senesce, and other researchers have also recognised resource limitation at the later stages of *P. somniferum* development (Chung 1982, 1987). While hormonal cues from established fruit and seed are also known to affect resource allocation to other sinks, and plant growth regulators produced by developing fruit may prevent future bud development (Stephenson 1981, Stephenson 1992), this area has not been widely researched in poppies (Pontorich and Sedova 1976, Pontorich *et al.* 1984) and there is little in this study that may further elucidate these processes.

For 25% of the plants upon which bud senescence occurred, the senesced bud was not at the last branch position and reproductive success was evident on the next branch to develop, e.g. a plant produced capsules at positions 1 and 2, bud senescence occurred at

position 3, and a capsule was produced at position 4. This often occurred on larger, more highly branched plants. The senescence of floral buds at different stages of plant development suggests that maternal resources fluctuate in availability during plant growth and that resource regulation within a plant varies temporally. This variation in the timing of bud senescence has been noted in other indeterminate species where there is sequential floral development (Lloyd 1980, Stephenson 1992, Stephenson 1984). In this study it appears that for some poppy plants, resources were limited early in plant growth, resulting in bud senescence midway through plant development. It can be assumed that once the resource demands of the previous capsules, seed and vegetative growth had been met; the flowering regime was reinstated. Many in this cohort of plants were large and highly branched, and early expansion of organs (vegetative and reproductive) may have quickly depleted maternal resources. In poppies, rapid growth of the stem is immediately succeeded by rapid capsule expansion, which is immediately succeeded by rapid seed development (Chung 1982). Further, the capsule development on successive branches overlaps (e.g. when flowering and fruiting at position 2 quickly follows that at position 1), and the resources required to support the sink strength of both developing fruits would be significant. This may trigger bud senescence at the next branch in order to postpone the development of another sink before adequate resources have been allocated to the capsules already present (Cawoy *et al.* 2007).

The patterns of biomass allocation and regulation of flower production of field grown poppies as observed in this study concurs with observations in other species (Morrison and Myerscough 1989, Shillo *et al.* 1984) and is supportive of the hypothesis that there is serial maternal investment in reproductive structures, where control of within-plant developmental processes is moderated by local resources (Lloyd 1980). For the poppy

plants in this study it appears that there were intrinsic controls influencing allocation and bud senescence, and that fruit production and yield were regulated within the plant. This may be the reason that there were no significant differences between sites for plant productivity, and why density (at the low levels recorded at the study sites) was not a significant predictor of within-plant capsule mass.

3.5.5 Capsule morphine yield

It is important to consider biomass allocation at the position-level in poppies, because it is the upper capsules that have the greatest pharmaceutical and economic importance as they contain the greatest amount of alkaloids. This has been shown in earlier research (Bhandari *et al.* 1988, Khanna and Gupta 1982, Shukla *et al.* 2004, Ogrodowczyk and Wawrzyniak 2007) and was supported by the results of the GLM on the subset data (section 3.4.5). The model revealed a significant difference in morphine concentration between capsule positions for three of the four sites, with the main capsule (capsule 1) generally containing the greatest morphine concentration (Figure 3.11). This result does appear to support a gradient in sink-strength and/or biosynthetic capacity between capsules (section 3.5.2). Indeed, Facchini *et al.* (2005, Facchini and Park 2003) described the differential biosynthetic gene expression linked to plant development in *P. somniferum* (high in capsules and stems, and lower/variable in roots and mature leaves), in agreement with other alkaloid accumulation studies (Williams and Ellis 1989), and it may be that developmental regulation of alkaloid synthesis also occurs between capsules.

It is notable that in this study the capsule concentrations were quite variable, also previously observed in the individual capsule concentrations of other work (Ghiorghiță

et al. 1990, Ogrodowczyk and Wawrzyniak 2007). The variability indicates that although capsule position was a significant factor in alkaloid yield, environmental variables such as light and temperature (Bernáth and Tétényi 1981, Chung 1987, Ghiorghiță *et al.* 1990) remain significant contributors to capsule alkaloid variability. This was demonstrated at site 1, where there was a lack of a significant difference between any of the positions in their capsule morphine concentrations (Figure 3.9).

An understanding of the biomass allocation patterns to capsules within poppy plants is important, as maximising capsule mass yields at the positions that contribute the majority of alkaloid yield could improve crop alkaloid yield. Further, although it may be argued that the gains in mass and alkaloid yield from the upper capsules could be offset by a dilution effect of the presence of lower capsules, this study showed that on highly branched plants the likelihood of bud senescence is high, therefore few lower capsules would be produced to dilute yield. In this study, the patterns of biomass allocation observed within the plant suggests that management of plants to increase the parameters important in capsule net mass of the upper positions would also increase seed mass and may have implications for alkaloid yield.

3.6 Conclusions and recommendations

This field study of a commercial poppy line elucidated patterns of within-plant biomass allocation not commonly investigated for *P. somniferum*. Previous work has described correlations between morphological traits in poppy but has failed to adequately capture how the plant developmental process and resultant branching architecture relate to within-plant biomass allocation patterns, an important consideration for the commercially relevant capsule positions. This study found that in field crops of poppies

there were indications of developmental, competitive and plant architecture effects regulating the allocation of biomass to certain capsule positions.

Considering the complex interplay of sink strength and feedback mechanisms, and with so little modelling of within-plant biomass allocation in poppies, it is difficult to fully clarify allocation processes and additional, controlled studies are warranted. It would be advisable and beneficial to investigate the effects of treatments including nutrients, water stress and planting density. A consideration of the integration between branches would also be informative, examining available leaf area, leaf duration and the effects of flower removal.

Also suggested is a closer investigation of the timing of flowering and bud senescence, and a microscopic examination of the senesced bud and ovary therein. Hormonal feedbacks in poppies have received scant attention and this is also an area worthy of investigation.

The improvement of alkaloid yield, although closely linked to the yield of capsule mass, is less certain due to the impact of environmental variables. In order to improve alkaloid yields beyond this, the accumulation of alkaloids in the capsule requires deeper investigation.

CHAPTER 4 The utility of Nuclear Magnetic Resonance (NMR) for the investigation of capsule laticifers in *Papaver somniferum* L.

4.1 Introduction

Alkaloid yields in *Papaver somniferum* L. have been linked to plant morphology, but are also affected by environmental variables. Considerable variations in both plant yields and capsule yields have been described even within the same genotype (Ghiorghiță *et al.* 1990); the implication being that yield is not determined solely by the plant form, but that other factors also contribute to the accumulation of alkaloids in the capsule (section 3.4.5). In order to fully explore means of yield improvement in *P. somniferum*, these processes need to be understood.

It has been demonstrated that the alkaloid yield in capsules is affected by temperature, light, sowing date, irrigation and nutrient application (Bernáth and Tétényi 1981, Chung 1987, Laughlin 1987) and throughout many studies there is also the suggestion that alkaloids synthesised in various parts of the poppy plant are translocated to the capsule during plant development (Nikonov 1958). Whilst there have been some dedicated translocation studies, there is little evidence to demonstrate that latex translocation is possible and, if it does occur, what may be the governing mechanisms.

To investigate translocation it is necessary to study the laticifers, the cells in plants that contain latex. It has been demonstrated that the capacity of laticifers is fixed per unit tissue (section 2.4, Figure 2.2). So although laticifers do not contribute to yield

variability through differences in structural capacity, they may have a functional impact through a role in substance transport.

The alkaloids in opium poppy are sequestered to vesicles within poppy latex, a substance that is exclusively stored in the reticulated network of laticifers distributed throughout the plant. Laticifers are located within the vasculature of the plant and range from 10-50 μm in diameter. These cells have hydrated primary cell walls composed of cellulose, hemicellulose and pectins (Fahn 1989) and the connections between cells occur through enzymatic dissolution of the cell walls (Nessler and Mahlberg 1977b). Laticifer cell walls are also highly elastic and latex within these cells is stored under pressure, a feature that renders it difficult to microscopically investigate laticifers without significant disruption to cells. Thus, it is necessary to employ extensive tissue fixing and staining techniques for cellular observation, which may compromise tissues (Ruzin 1999, Nessler and Mahlberg 1981), and also provides information that is limited to that particular tissue section.

Nuclear magnetic resonance (NMR) microscopy is a technique that has gained utility in the study of plant cellular distribution and physiology due to the ability to work non-invasively and to perform repeated, quantifiable measurements on intact plants (with little preparation). It has previously been employed in the investigation of a variety of fruits including; raspberry, blackcurrant, grape and apple (Goodman *et al.* 1992, Glidewell *et al.* 1997, Glidewell *et al.* 1999, Ishida *et al.* 2000). There has been demonstrated success in imaging details of fruit tissue arrangement in both 2D and 3D; the anatomical development from flower to fruit; and Glidewell *et al.* (1997) imaged the spread of fungal infection in grapes. The range of applications is more extensive than

this however, with NMR techniques also used to image both short and long distance water transport (Windt *et al.* 2006, Manz *et al.* 2005, Garnczarska *et al.* 2007, Ilvonen *et al.* 2001), solute concentration and solute flow (Köckenberger *et al.* 2004, Verscht *et al.* 1998), for the characterisation of cell growth (Snegireva *et al.* 2006), and to study seed maturation and imbibition (Morris *et al.* 1990). Therefore, the technique of NMR imaging can extend beyond microscopic anatomical descriptions to also inform on plant metabolism and physiology, and as such may be of use in the investigation of latex translocation.

NMR is based on the detection and interpretation of a signal received from target nuclei within cells, typically the hydrogen-bound nuclei of water. Placing the sample within a strong, static magnetic field and then applying a linear oscillating field generates the NMR signal (Köckenberger *et al.* 2004). Nuclei that have the property of spin will align with the strong magnetic field, and a proportion of the population of nuclei will be energised to a higher energy state if the oscillating field is at the correct frequency (the Larmor frequency). A detectable weak voltage is generated by the differences in energy states and it is this voltage that constitutes the NMR signal from the two-dimensional (pixel) or three-dimensional (voxel) area being imaged. The production of an image from this signal is possible as the linear gradient of the oscillating field (perpendicular to the static magnetic field) allows for spatial encoding through Fourier transformation (a mathematical transformation of time data).

Two relaxation processes occur simultaneously during NMR of a sample: spin-lattice relaxation (T1) and transverse relaxation (T2). T1 is the time taken for nuclei to return to their initial state of equilibrium after excitation and is a dissipative process involving

energy exchange. T2 is the time taken for phase decoherence of spin packets (groups of nuclei with spin) and results in the decay of the net magnetization of a sample. Phase decoherence occurs because; a) macroscopically, each of the spin packets (which collectively produce an overall net magnetization) experience a slightly different magnetic field due to non-homogeneities across a biological sample, e.g. air and water respond to magnetisation differently which results in field distortion, and b) microscopically, through molecular interactions (Faust *et al.* 1997).

T1 and T2 provide a vast array of information on the nature of cellular contents; e.g. a longer T1 time indicates greater mobility of molecules and is a feature of actively metabolising tissues; and a greater concentration of solutes (viscosity) increases molecular interactions, thus decreasing T2 duration (MacFall and Johnson 1994). Additionally, differences in relaxation times have been shown to enable the differentiation of cellular compartments based on their water content (van der Weerd *et al.* 2002, Ratcliffe and Shachar-Hill 2001).

The intensity of the NMR signal is dependent upon both the density of spins and the relaxation times (van As 2007) so, when the hydrogen-bound nuclei of water is the NMR target, regions of tissue that have greater amounts of mobile water will produce a stronger signal. Also, because the image is constructed from the signal received from water, it is representative of the solution between structures (such as cell walls) and not the structure itself. This has the benefit of allowing not only the visualisation of cells, but to enable an investigation of cellular water content, water flow and cell metabolism in intact tissues (Ishida *et al.* 2000).

Based on the capacity of the NMR technique, laticifers, which contain a complex, viscous latex and multitudes of vesicles, are a cell type that would provide a unique signal that would theoretically be distinguishable from the surrounding parenchyma and phloem. Indeed, it has been demonstrated that NMR methods may have sufficient resolution to visualise cells with large diameters (30-100 μm) in order to clearly describe vascular anatomy and cellular patterns (Köckenberger *et al.* 2004). The laticifers of *P. somniferum* L. have been extensively described using the conventional techniques of bright field microscopy and electron microscopy (Fairbairn and Kapoor 1960, Nessler and Mahlberg 1977a, 1977b, 1978, 1979i, 1979ii, 1981). These studies have been able to provide static anatomical information, to describe laticifer development and reticulation during the maturation of poppy capsules, and have localised specific metabolites to cell types, yet they are limited in their ability to inform on latex translocation.

This investigation sought to assess the utility of nuclear magnetic resonance (NMR) microscopy to:

1. differentiate between xylem sap, phloem cell sap and laticifer latex
2. to achieve resolution sufficient to discern the cellular distribution of capsule vascular bundles, and subsequently to
3. map the laticifer architecture and content in a maturing poppy capsule without disruption to tissues, in an attempt to better understand translocation.

4.2 Materials and Methods

Method development was conducted at the Central Science Laboratory (CSL) of the University of Tasmania, Australia. The CSL is equipped with a wide bore Varian 'Inova' 400 MHz spectrometer that is capable of micro-imaging, using one of 4 interchangeable probe coils. This study used the largest diameter, 30mm. Both ^1H NMR and chemical shift imaging were attempted.

4.2.1 Glasshouse conditions

Capsule samples were sourced from a high morphine-producing line of *P. somniferum*, grown in glasshouse conditions. Seeds were sown in six-inch pots on potting mix containing slow release fertiliser and were covered with fine vermiculite. Dolomite lime was added to the potting mix to control pH to 6.5. Irrigation was provided twice daily using a capillary matting system, reducing the incidence of fungal attack. Additionally, plants were subjected to a 16 h extended daylight regime, liquid fertilised fortnightly and received weekly applications of foliar calcium nitrate (5 gm L^{-1}) to overcome male sterility. Plants were tagged with dated labels on the day that flowers opened, and flowers were manually pollinated using a petal as a pollen applicator.

4.2.2 Sample preparation for NMR

Two weeks after flowering, a sample of poppies was heated-treated with a miniature, compressed gas burner (to coagulate latex) at a point on the stem 20 cm below the capsule. Plants were cut at this point, and the upper portion of the stem holding the capsule was placed in a bag and refrigerated until use later that day. Intact plants were

also prepared; potting mix was washed from the roots, moistened cotton wool was wrapped around the root ball that was then placed in a plastic bag and secured with elastic bands. Parafilm™ was tightly wrapped around the enclosed roots to ensure that the sample was water-tight. Each capsule sample was inverted into the spectrometer bore, suspended, and ^1H NMR images of the whole poppy capsule were captured through the transverse plane. The slice selection was set at 0.235 mm and in-plane resolution was 25x50 μm . A recycle time of 5 sec was used with a spin echo time of 12 ms.

Conventional transverse sectioning was also performed on two-week-old capsules, with samples being lightly stained with Toluidine Blue and observed using a Zeiss ‘Axioscop 2 plus’ microscope fitted with an ‘AxioCam HRc’ digital camera for image capture.

4.3 Results

The typical distribution of tissues in the vascular bundles of the poppy capsule is illustrated in the light micrograph of a transverse section of a capsule (Figure 4.1).



Figure 4.1. Light micrograph of transverse section of poppy capsule. L = laticifer, P = phloem, X = xylem. Scale bar = 30 μ m.

The xylem is typically located proximally to the inner capsule, with the phloem arranged peripherally. The latex-containing laticifers are located on the outer side of the phloem and in this image constitute a large proportion (one third) of the area of the vascular bundle. It is the arrangement and the amount of tissue given to laticifers that suggest that the use of NMR may be an appropriate tool for the investigation of the laticifers within a capsule.

4.3.1 Visualisation of laticifers; H^1 NMR images

H^1 NMR images of serial transverse sections are presented in Figure 4.2. The series begins at the bottom right of the image, starting with images from the poppy stem, through the thalamus at the base of the capsule and then a series of transverse sections up through the capsule. There are 11 major vascular bundles, evident as bright points around the stem periphery (images on the lower segment of Figure 4.2). These continue up through the mesocarp of the capsule, becoming the placental bundles that then terminate in the stigmatic rays (this capsule had 11 stigmatic rays). Minor trace bundles are located in the outer mesocarp, but are indistinguishable in this image. Poppy seeds are located in the outer mesocarp, but are indistinguishable in this image. Poppy seeds are evident in great numbers in the capsule, producing a granular effect within the capsule. The seeds fill the areas between the placental rays, which extend toward a central space (Figure 4.2). The photograph of a sectioned capsule in Figure 4.3 clearly illustrates the arrangement of placental tissue with seeds filling spaces between, and an area in the centre of the capsule that is devoid of tissue.

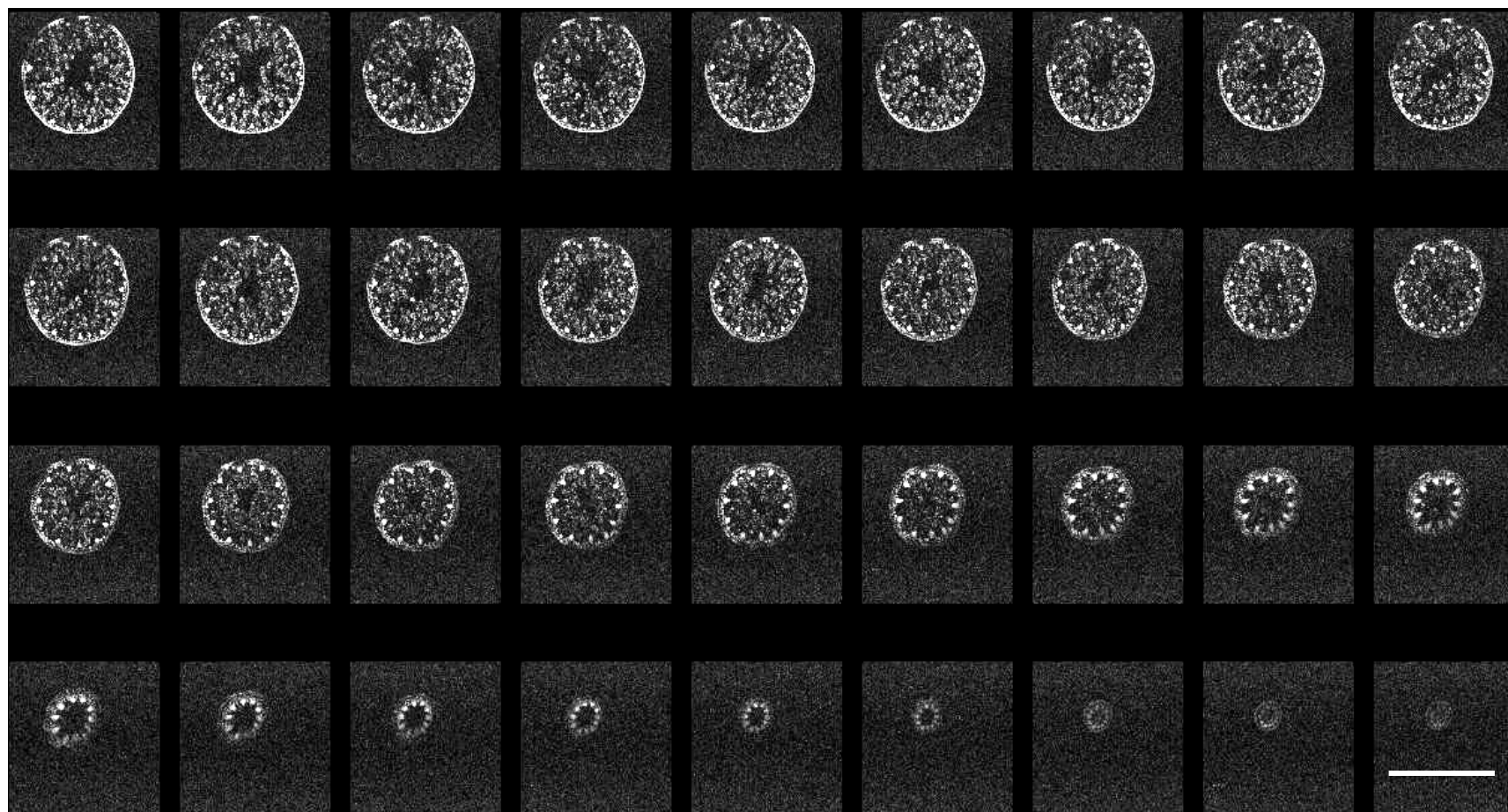


Figure 4.2. H^1 NMR images of serial transverse slices through a poppy capsule. Slice selection was set at 0.235 mm and in plane resolution is $25 \times 50 \mu\text{m}$. A recycle time of 5 sec was used and an echo time of 12 ms. Scale bar = 3 cm.

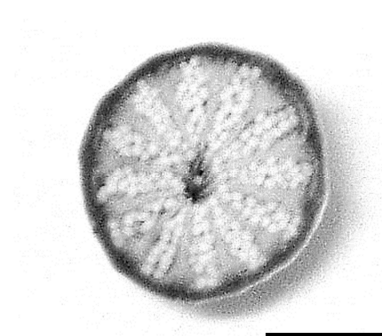


Figure 4.3. Transverse section of a poppy capsule, *Papaver somniferum*. Scale bar = 3 cm.

4.3.2 Visualisation of laticifers; contour plots

The ^1H NMR image in Figure 4.4 is presented as a contour plot, with the signal of water depicted in 'hotter' colours as the signal strength increases (McCain and Markley 1992). This image represents a fraction of a transverse section of the outer part of a capsule, sampled at two weeks after flowering. Three major vascular bundles are visible in the mesocarp around the perimeter of the capsule (one is circled in white). These are the placental vascular bundles and the smaller bundles in the outer mesocarp are the trace bundles. In the circled vascular bundle, the area of xylem tissue is apparent as red and orange (located proximally in the bundle as described in the light micrograph, Figure 4.1) with phloem and laticifers as the blue/green region (refer Figure 4.5 for vascular bundle detail).

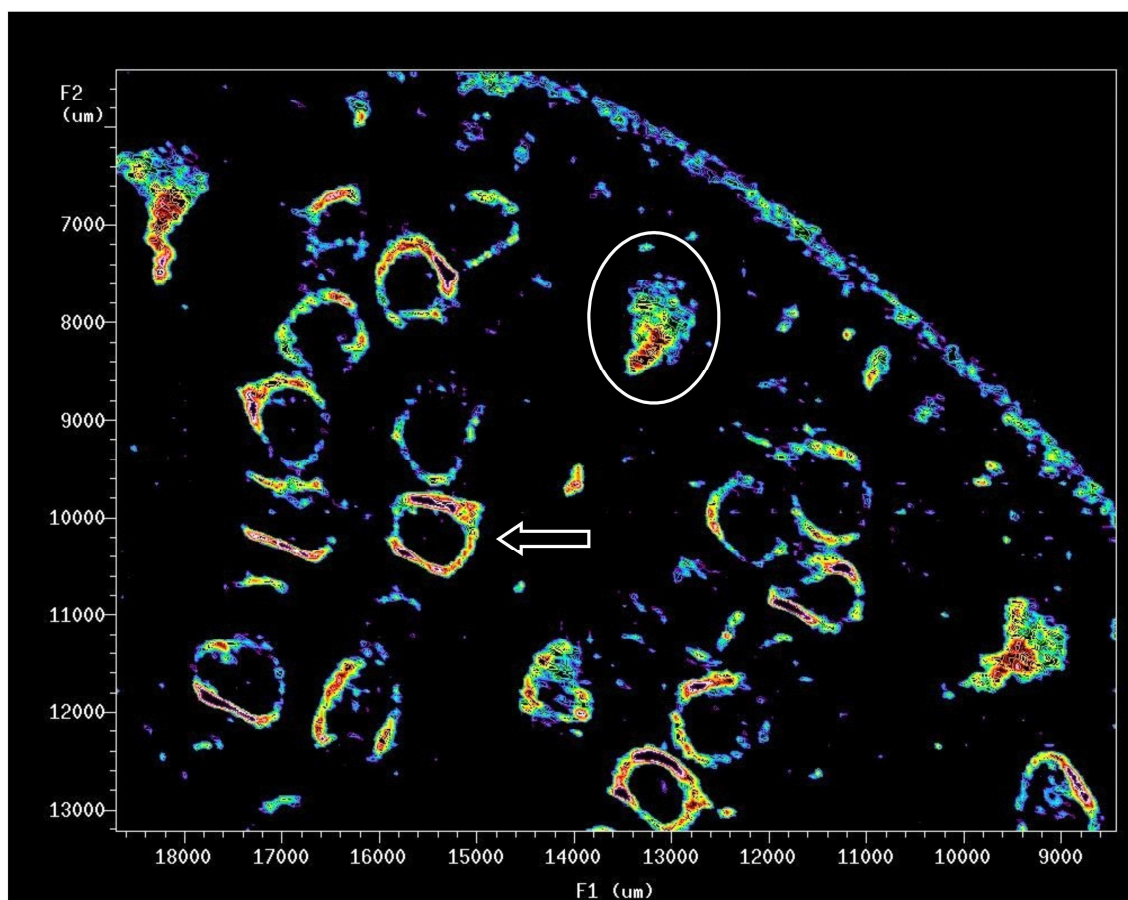


Figure 4.4. H^1 NMR image of whole poppy capsule through transverse plane, presented as contour plot. Slice selection was set at 0.235 mm and in plane resolution is $25 \times 50 \mu\text{m}$. A recycle time of 5 sec was used and an echo time of 12 ms. A placental vascular bundle has been circled in white and the block arrow is marking a seed.

The other coloured, circular structures visible are the developing seeds (block arrow), which are located along the placental rays (the predominantly black regions between the rows of seeds). Note the very strong signal from the seed testa. The minor bundles of the mesocarp (trace bundles) are evident around the periphery.

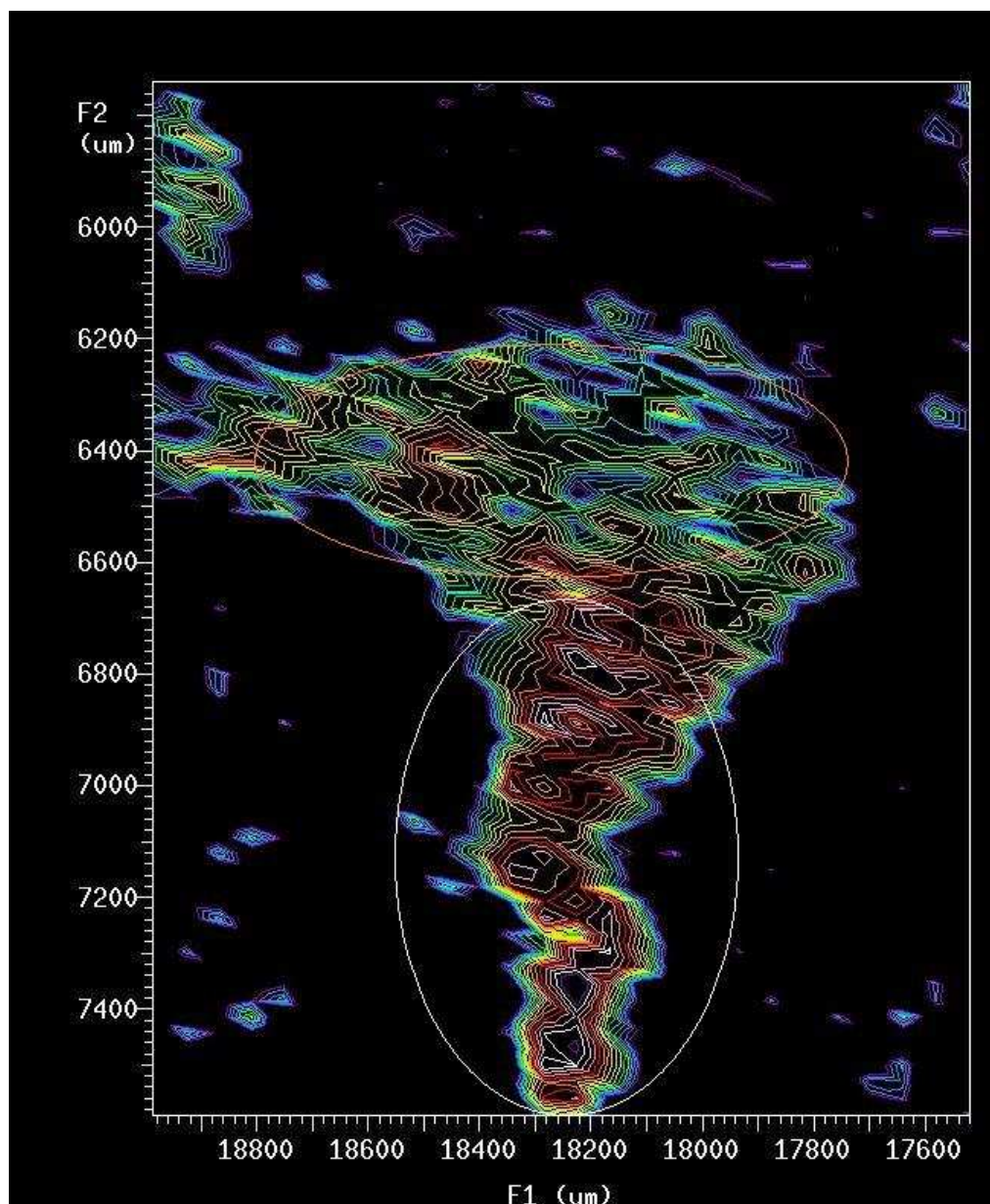


Figure 4.5. H^1 NMR detail image vascular bundle in poppy capsule, taken through transverse plane, presented as contour plot. Slice selection was set at 0.235 mm and in plane resolution is $25 \times 50 \mu\text{m}$. A recycle time of 5 sec was used and an echo time of 12 ms. Xylem tissue is circled in white and phloem/laticifers are circled in orange.

Figure 4.5 depicts a contour plot detail of a capsule vascular bundle, with xylem being interpreted as the area producing the strongest signal (red and orange), and phloem and laticifers as the blue/green region.

4.4 Discussion

This trial investigated the utility of NMR micro-imaging in visualising the laticifers of *P. somniferum* capsules. There was little success in imaging capsules using chemical shift methods, however the use of ^1H NMR produced images in which it was possible to discern the seeds and the placental tissue (Figures 4.2, 4.4 and 4.5), the major and minor vascular bundles, and areas that can be interpreted as xylem and phloem within the vascular bundle. Dark areas were apparent within the centre of the capsule, in each seed (Figure 4.4) and no signal was detected from the placental tissue in either the ^1H NMR series or the contour plot (Figures 4.2 and 4.4). It was also possible to trace the course of vascular bundles from the stem, through the thalamus and into the capsule (Figure 4.2).

Areas of low, or no, signal from the placental tissue have been noted by other researchers such as Glidewell *et al.* (1999), who also identified the dark areas in blackcurrant fruit as placentae. There are several reasons for a lack of signal in NMR studies. The magnetic distortion produced by air-water interfaces in tissue, especially where there are many small cells present, results in rapid dephasing and thus signal loss (Faust *et al.* 1997). Alternatively, areas where no tissue is present will also appear dark (as in the center of the capsule), however this is not applicable here because placental vascular bundles are evident (Figure 4.3). It is more probable that the placental tissue appeared as a dark region due to low proton mobility in comparison with other nearby tissue areas, such as the seed testae (Figure 4.3).

Prokof'ev *et al.* (1984) described the osmotic and water potential concentration gradients between the seeds and placentae in poppy capsules, and it is probable that the placental tissue, with a greater concentration of osmotically active constituents (such as sugars) at this point in development, has low proton mobility due to molecular interactions. This would result in a shorter T2 relaxation time (phase decoherence) with spin packets quickly returning to their state of equilibrium, thus reducing the signal from this part of the capsule tissue. MacFall and Johnson (1994) were also able to demonstrate the effect of sucrose concentration in altering the H^1 signal, through visualising dark areas around the vasculature of sugar beet and identifying these as storage tissue.

Within the seeds dark areas are also apparent, however this is more likely a result of the high concentration of lipids, which have been shown to reduce relaxation times (Faust *et al.* 1997, Goodman *et al.* 1992, Connelly *et al.* 1987). Goodman *et al.* (1992) studied raspberry seed and found that when in the context of the fruit, the seed did not produce a signal, but when investigated as an individual seed, mobile protons were detected. These authors attributed the lack of signal to the lower concentrations of mobile protons in the seeds compared to the mesocarp. Connelly *et al.* (1987) stated that it is common for low intensity signals to be generated by the seed interior and indicates that the lack of signal is a useful feature for discerning structures within fruit. This is especially relevant in the current study, where such a vivid contrast existed between the non-imaged seed interior and the signal received from the seed coat. In fact, the intensity of the seed coat was very similar to that of the xylem in the major vascular bundle of the placenta, and thus provides an excellent indication of the comparative water status of the seeds at the capsule age of two weeks (post anthesis). Prokof'ev *et al.* (1981)

examined the water exchange of ripening poppy capsules and compared the water status of seed, placenta and mesocarp, but the application of NMR in this regard represents an advance, and has implications for the rapid assessment of water relations in intact, developing poppy fruit.

This is further highlighted by the detection of the minor bundles of the mesocarp (trace bundles), which were evident around the capsule periphery (Figure 4.4). Previous authors also detected minor bundles (Glidewell *et al.* 1999), however the current study achieved greater image resolution and was able to differentiate between xylem and phloem within a vascular bundle. It was not possible, however, to achieve the requisite resolution for the differentiation of laticifers and phloem elements (Figure 4.5).

The quality of plant material was not a contributing factor in this study. It could be suggested that because the images were produced from an excised stem the water status of the sample may have suffered, but the strong signals from the vascular bundles and seed coats indicates that hydration was sufficient. An alternative possibility is that the non-homogeneity of capsule tissue may present an issue for imaging. While other studies have had success in imaging consistent plant tissue, it may be that the greater complexity of the sample hindered the discrimination of laticifers from phloem in the presence of the strong signal received from xylem in the vascular bundles. Additionally, while it is possible to increase resolution through increasing acquisition times (Windt *et al.* 2006), it would not be feasible to make further increases on the duration already applied without compromising sample function. As the sample was suspended within the bore of the NMR, the inability to supply sufficient lighting would

compromise photosynthetic ability. Some of the restrictions for an investigation of translocation then, are attributable to the capability of the available facilities.

As a result of this trial, although it was not possible to identify laticifers within the vascular bundle and thus investigate translocation in this cell type, it was possible to trace the course of vascular bundles from the stem, through the thalamus and into the capsule, confirming the existence of a contiguous pathway from poppy stem to stigmatic ray.

4.5 Conclusion

This is the first time the vasculature of poppy capsules has been imaged using the non-invasive method of NMR. Using this technique it was possible to trace the course of vascular bundles through the thalamus of the stem into the capsule and to discern tissue areas within a vascular bundle, which represents an advance on previous research into fruit vascular anatomy. Using this method of NMR, however, it was not possible to achieve the resolution needed to clearly distinguish between cell types within the vascular bundle. Thus, it was not possible to identify laticifers in a capsule, to ascertain the volume of capsule tissue given to laticifers, or to monitor fluctuations in latex. NMR does have the potential to be a very useful tool for an investigation of capsules in poppy, but due to the limits of the facilities and issues of resolution, the utility perhaps lies in developmental studies and investigations of water relations.

CHAPTER 5 Vesicle populations in the capsule latex and the stem latex of *Papaver somniferum* L. – implications for capsule development and latex translocation

5.1 Abstract

This research investigated the vesicle populations of capsule latex and stem latex of *Papaver somniferum* L. In three separate studies, vesicle populations were examined during plant development, at localised positions, and in terms of their volume. It was established that capsule latex contained significantly greater vesicle numbers and had a greater packed vesicle volume (PVV) than did stem latex, and that this difference occurred as a steep gradient in the thalamus of the poppy capsule. Further, the studies demonstrated that for two of the three positions sampled, latex vesicles increased in number during plant development, but that the pattern of development varied according to position on the plant. It was suggested that the most parsimonious explanation of the observed vesicle populations and vesicle gradient is that of *in situ* vesicle production, and that the translocation of vesicles through the bulk latex flow, or *in toto* movement, is dubious.

5.2 Introduction

Although laticifers do not contribute to variability in capsule alkaloid yield through differences in structural capacity (section 2.4), they may have a functional impact through a role in latex transport. Laticifers of *Papaver somniferum* L. are located between the phloem cells of vascular bundles and akin to the phloem sieve cells, the content of laticifers (latex) is under pressure. Translocation of assimilates in phloem occurs through sieve cells from source to sink, and in the plant this may be in both directions (up and down) and also laterally (van Bel 2003, Eschrich 1975). Several authors have reported that there is also translocation of latex in the laticifers of poppies (Fairbairn *et al.* 1964, Fairbairn and El-Masry 1967, Fairbairn *et al.* 1973, Fairbairn *et al.* 1974, Vágújfalvi 1966), resulting in the transport of alkaloids to the capsules where these substances concentrate over the period of capsule development. Although the results of these authors (especially Fairbairn and El-Masry 1967 and Fairbairn *et al.* 1974) appear to suggest that translocation of latex occurred, due to the inherent issues of experimental inference and possible experimental contamination in these studies, there is an imperative to conduct further investigations that address the possibility of latex movement in a contained, pressurised system such as the laticifers of *P. somniferum*.

It was not possible to directly visualise laticifers or their contents in Chapter 4, however, it may be possible to infer the occurrence of translocation through an investigation of vesicles in the latex of the capsule and stem of poppies. Bulk movement of latex from stem to capsule would also move the content of vesicles toward the capsules (Fairbairn *et al.* 1974), and thus investigations of vesicles could provide information regarding the movement of vesicle populations through the plant.

Poppies produce alkaloids as a consequence of secondary metabolism, and these metabolites are sequestered into the vesicles of laticifers. Vesicles are present in large quantities in latex, especially during the latter stages of capsule development (Griffing and Nessler 1989, Nessler and Mahlberg 1977a, 1977b, Thureson-Klein 1970) and are heterogeneous in size, reported as 0.3 μm to 3 μm in diameter (Decker *et al.* 2000, Thureson-Klein 1970). Early research indicated that vesicles originated from the endoplasmic reticulum (ER), with observations that ER membranes were prolific early in laticifer development but were fewer in number later in development, when vesicles constituted the majority of latex (Thureson-Klein 1970). Later, Nessler and Mahlberg (1977a) proposed a developmental pathway for alkaloid vesicles whereby dilations of the endoplasmic reticulum (with ribosomes) gave rise to vesicles, which the authors substantiated through cytochemical evidence. Although Griffing and Nessler (1989) subsequently proposed an alternate ontogeny for vesicles, recent research appears to support the ER origin (Alcantara *et al.* 2005, Amann *et al.* 1986, Bock *et al.* 2002).

Two forms of vesicle have been identified in poppy; the first having a granulated outer membrane, and the second comprising the heavier fraction of latex (centrifugally) and observed with both 'caps' and an ordered interior (Dickenson and Fairbairn 1975). Nessler and Mahlberg (1979i) suggested that the electron dense particles that accumulate in vesicles are composed of lipoprotein. Subsequent microscopical studies did not identify the same features apparent in capped vesicles, but were able to relate particular morphological features to different vesicle fractions (Fairbairn and Steele 1981, Roberts *et al.* 1983), and agreed that particle inclusions were not alkaloids (Roberts *et al.* 1983).

The majority of research on latex has investigated both the supernatant and the subpopulations in vesicles through density fractionation of the latex, and there is some contention regarding the localisation of biosynthetic enzymes, alkaloids and proteins. It has been suggested that the supernatant contains biosynthetic enzymes (Decker *et al.* 2000, Kamo and Mahlberg 1984, Roberts *et al.* 1983), unidentified protein (Kamo and Mahlberg 1984), and major latex protein (MLP; Decker *et al.* 2000), yet others have identified these in vesicle subpopulations (Fairbairn and Djoté 1970, Griffing and Nessler 1989, Roberts *et al.* 1983). Both Griffing and Nessler (1989) and Kutchan *et al.* (1986) cite differential vesicle fragility, which may explain some of the discrepancies in substance localisation.

Additionally, many of these authors have identified subpopulations of vesicles within latex, based on their alkaloid content. Griffing and Nessler (1989) identified subpopulations for morphine, and for dopamine; Roberts *et al.* (1983) localised morphine, codeine and dopamine to the heavy fraction; and Kutchan *et al.* (1985) and Kutchan *et al.* (1986) found that dopamine, sanguinarine and thebaine were contained in vacuoles of different densities, but for *Papaver bracteatum*. Subpopulations have also been described in terms of their ability to absorb morphine and respond to temperature (Roberts *et al.* 1991), and in their accumulation of alkaloids during plant development (Pham and Roberts 1991).

Pham and Roberts (1991) in their investigation of poppy alkaloid sequestration demonstrated that the volume of alkaloid vesicles in latex (and the concentration of morphine therein) increased over a period of capsule development (weeks 0-3 after anthesis), and that the nature of the vesicles also changed. The heavier vesicle fraction

(900 xg) was found to constitute a greater proportion of the latex in the weeks after flowering than did the lighter fraction (1100 xg), but the authors were not able to identify whether this was a result of an increase in vesicle number or an increase in vesicle size. Decker *et al.* (2000) suggested that density relates to developmental state, which is in agreement with the observation by Pham and Roberts (1991) that the heavier fraction increased over the monitored developmental period, and that the majority of alkaloids are located in the dense fraction of latex, which concurs with Roberts *et al.* (1983).

These studies provide significant information regarding latex content and activity, yet several issues are unresolved and there is no clear indication that bulk (*in toto*) latex movement occurs. The objectives of this investigation were to:

1. enumerate the vesicle populations in latex sourced from several positions in developing poppy plants
2. assess the vesicle population at the capsule-pedicel junction on the thalamus of the capsule, in terms of both number and volume, and
3. ascertain whether latex vesicles are produced *in situ* in the capsule and/or whether they are translocated to the capsule.

5.3 Materials and methods

5.3.1 Glasshouse conditions

Plants of a high morphine-producing line of *P. somniferum* were grown under controlled glasshouse conditions. Seeds were sown in six-inch pots on potting mix containing slow release fertiliser and were covered with fine vermiculite. Dolomite lime was added to the potting mix to control pH to 6.5. Irrigation was provided twice daily using a capillary matting system, reducing the incidence of fungal attack. Additionally, plants were subjected to a 16 h extended daylight regime supplied by 400W mercury vapour lamps, they were liquid fertilised fortnightly and received weekly applications of foliar calcium nitrate (5 gm L⁻¹) to overcome male sterility. Plants were tagged with dated labels on the day that flowers opened, and flowers were manually pollinated using a petal as a pollen applicator.

5.3.2 Sampling methods

5.3.2.1 Method development – buffer selection

As there were a number of collection buffers described in the literature on poppy (Decker *et al.* 2000, Kutchan *et al.* 1986, Nessler *et al.* 1985, Pham and Roberts 1991, Pilatzke-Wunderlich and Nessler 2001, Roberts *et al.* 1983) an assessment of buffers was undertaken to ensure that the vesicle population of the latex was successfully maintained; that lysis and agglutination were precluded, producing the optimal buffer for vesicle enumeration.

A trial of 0.1 M potassium phosphate buffers ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) with three varying concentrations of mannitol was conducted to assess the efficacy of 250 mMol, 500 mMol and 750 mMol (mannitol) at maintaining an intact vesicle population. Buffers were made to a pH of 7.1 at room temperature.

The capsules were excised from fully turgid poppy plants and 10 μL of exuded latex was immediately collected from the base of the capsule using a Drummond Wiretrol[®]1 micropipettor. The latex sample was expelled into a labelled Greiner tube containing 20 mL of ice-cold buffer, gently spun using a bench vortex to disperse latex without vesicle disruption, and placed on ice. Diluted latex was pipetted into an haemocytometer and, for each sample, was visualised using the Diffusion Interference Contrast (DIC) mode on a Zeiss 'Axioscop 2 plus' microscope fitted with an 'AxioCam HRC' digital camera.

In order to quickly process the samples, images were captured of five haemocytometer squares (four corner squares and the middle square) for each sample, for later assessment. Counts of vesicles were undertaken using Image J Launcher software (v 1.4.3) and the number of vesicles per millilitre latex was calculated, accounting for latex dilution, the number of squares counted and haemocytometer volume.

It appeared that the population of vesicles was best maintained when diluted with 0.1 M potassium phosphate buffer containing either 250 mMol or 500 mMol mannitol, although when a one-way ANOVA test was applied there was no statistically significant difference ($F = 0.775$; $P = 0.502$). However, due to generally higher counts and the observation that agglutination occurred in the buffer containing 750 mMol mannitol

(Figure 5.1) only the concentrations of 250 mMol mannitol or 500 mMol mannitol in 0.1 M potassium phosphate buffer were deemed satisfactory for vesicle enumeration.

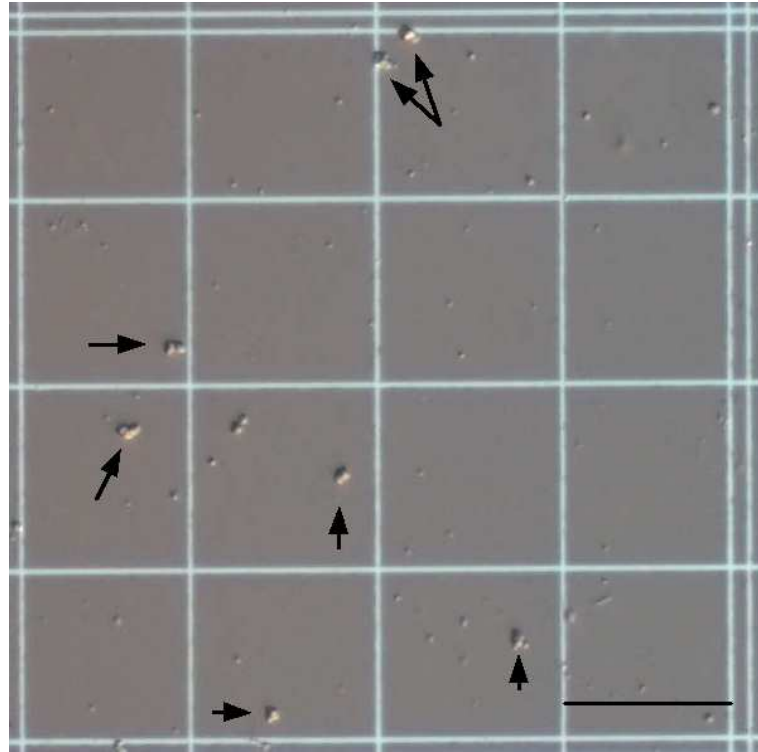


Figure 5.1. An image of one haemocytometer square containing vesicles from capsule latex collected into 750 mMol mannitol phosphate buffer. Arrows indicate clumped vesicles that are unacceptable for accurate counts. Scale bar = 50 μm .

5.3.2.2 *Enumeration of vesicles in latex at seven developmental stages*

The vesicle population in poppy latex was investigated at three positions in the plant and at seven developmental stages. Latex was sampled from the base of the stem, the top of the stem (pedicel) 10 cm below the capsule and above the uppermost leaves, and from the apex or capsule of the plant, depending on the stage. The stages investigated were: basal rosette (br), running up (ru), hook, flowering (f), seven days after flowering (f+7), eleven days after flowering (f+11), and fourteen days after flowering (f+14). At basal rosette stage the stem and apex were not present, and at running up stage only the base and apex were sampled due to plant size and latex availability. Sampling (for all investigations) was destructive and as a consequence, different plants were sampled at each developmental stage. Four plants (replicates) were sampled at each of the basal rosette and running up stages and all later stages had six replicates, a total of 38 plants for this study.

Poppy plants, when grown to the appropriate stage, were sampled in the early morning when fully turgid. Latex sampling was conducted when plants were fully hydrated to avoid the diurnal fluctuations previously observed in latex water content (Itenov *et al.* 1999), thus ensuring accurate enumeration and volume determination of vesicles. Plants were sectioned at each position using a Gem blade and 10 μ L of exuded latex was immediately collected from the cut using a Drummond Wiretrol[®]1 micropipettor. A new micropipettor was used for each position. The latex sample was expelled into a labelled Greiner tube containing 20 mL of ice-cold buffer and after being gently vortexed, was placed on ice. Sample loading onto the haemocytometer and vesicle assessment were performed as described in section 5.3.2.1.

5.3.2.3 Enumeration of vesicles in latex at the capsule-stem interface

A further study investigated the differences in vesicle populations of the capsule latex and stem latex. When six glasshouse-grown plants (replicates) were at f+7, latex samples were taken from both sides of a single cut made through the zone of abscission (of sepals and petals) on the thalamus between the capsule and the stem. A sample of latex was taken from one side of the cut then a second, separate sample was removed from the other side, effectively sampling capsule latex and stem latex individually and without moving stem latex into the capsule. The main axis (bearing the primary capsule) and first lateral branch (with secondary capsule) were sampled. Latex was collected using a 10 μ L Drummond Wiretrol®1 micropipettor, immediately expelled into a labelled Greiner tube containing 20 mL of ice-cold buffer, vortexed and placed on ice. Sample loading onto the haemocytometer and vesicle enumeration were conducted as described in section 5.3.2.1. For this study a 500 mMol mannitol 0.1 M potassium phosphate buffer was used.

5.3.2.4 Assessment of the volume of vesicles in latex at the capsule-stem interface

After enumerating the vesicles in the latex of capsules and of stems, the volume of latex occupied by vesicles was assessed. This was investigated to determine whether vesicle number affected vesicle volume in latex, or whether the volume of vesicles remained constant and vesicle size was significantly variable. The volume of vesicles in latex was determined for capsule latex and for stem latex, and at two developmental stages; f+7 and f+11. Five plants (replicates) were sampled at f+7 and four plants were sampled at f+11, nine plants in total. 500m Mol mannitol 0.1 M potassium phosphate

buffer was used for latex collection, at a 1:1 v/v ratio of latex to buffer (as per Decker *et al.* 2000, Nessler *et al.* 1985, Pham and Roberts 1991).

The volume of latex occupied by vesicles was determined centrifugally using a TOSCO centrifuge fitted with an hematocrit head. Fully turgid poppy plants at f+7 and f+11 were sampled early in the morning, and all samples were taken on the same day. Capsule latex and stem latex of the main axis of poppy plants was collected from one cut as described in section 5.3.2.3. Latex was collected using a Drummond Wiretrol®1 micropipettor and expelled into a labelled Eppendorf tube. The same volume of ice-cold buffer was immediately added and the sample was vortexed to mix. Eppendorfs containing latex/buffer samples were placed on ice until all nine plants had been sampled.

Once collection was complete, each sample was drawn by capillarity into a separate microhaematocrit tube (plain – non-heparinised), GMBH + CO KG ISO12772, $L = 75 \pm 1.00$ mm, $D = 1.55 \pm 0.05$ mm. The tube was stoppered with clay and placed in the haemaocrit head, noting the positions of samples, and centrifuged at 6000rpm for 5 minutes. After each tube was removed from the chamber following centrifugation, the packed vesicle volume (PVV) and the total volume were assessed using an MSE microhaematocrit reader. The total volume reading was corrected for the additional volume of buffer and the vesicle pellet in latex was expressed as the proportion of PVV in latex volume (LV).

5.3.3 Data analysis

5.3.3.1 Method development – buffer selection

Data normality was examined using an Anderson-Darling test in Minitab 11.12. As the data were not significantly different from a normal distribution (A-squared = 0.196, $P = 0.841$ with $n = 9$), it was deemed that a one-way ANOVA was appropriate to assess the effect of mannitol concentration (in 0.1 M potassium phosphate buffer) on the maintenance of the vesicle population in latex. This was conducted in SPSS v17. Homogeneity of variances was supported by the Levene's test ($P = 0.170$), illustrating that there was no significant difference in variance, and the one-way ANOVA conducted revealed no significant difference between mannitol concentrations ($F = 0.775$, $P = 0.502$ with $df = 8$). Concentrations of 250 mMol mannitol or 500 mMol mannitol were regarded as satisfactory for further experimentation.

5.3.3.2 Enumeration of vesicles in latex at seven developmental stages

Data were not normal and variances were heterogeneous, so data were transformed using a square root function. This normalised data (Anderson-Darling test; A-squared = 0.511, $P = 0.191$, $n = 101$) and stabilised the variances (Levene's test; $P = 0.789$). Transformed data were then analysed in SPSS v17.0, accounting for the sampling at three positions on one plant by denoting 'plant' as a random factor. Data were analysed using a general linear model (GLM) capable of analysing unbalanced data, with 'position' and 'stage' as fixed factors. Tukey HSD was employed for *post hoc* analysis.

5.3.3.3 Enumeration of vesicles in latex at the capsule-stem interface

As the data were normally distributed as assessed by an Anderson-Darling test for normality (A-squared = 0.260, P = 0.679, n = 24) and variances were stable (Levene's test; P = 0.995), analysis was undertaken by using a GLM in SPSS v17.0, with 'position' as a fixed factor and 'plant' as a random factor.

5.3.3.4 Assessment of the volume of vesicles in latex at the capsule-stem interface

PVV was expressed as a proportion of the latex volume; therefore data were transformed using an arcsine function. Transformed data were normally distributed (Anderson-Darling test; A-squared = 0.292, P = 0.563, n = 17) and variances were stable (Levene's test; P = 0.235). Analysis of transformed data was performed in SPSS v17.0, using a general linear model (GLM) incorporating the factors 'position', 'stage' and 'plant'.

5.4 Results

5.4.1 *Enumeration of vesicles in latex at seven developmental stages*

The number of vesicles in latex samples of the developmental study ranged from 5×10^9 vesicles per millilitre of latex to 2.27×10^{11} per millilitre of latex, with vesicle size approximately 2 μm in diameter (Figure 5.2).

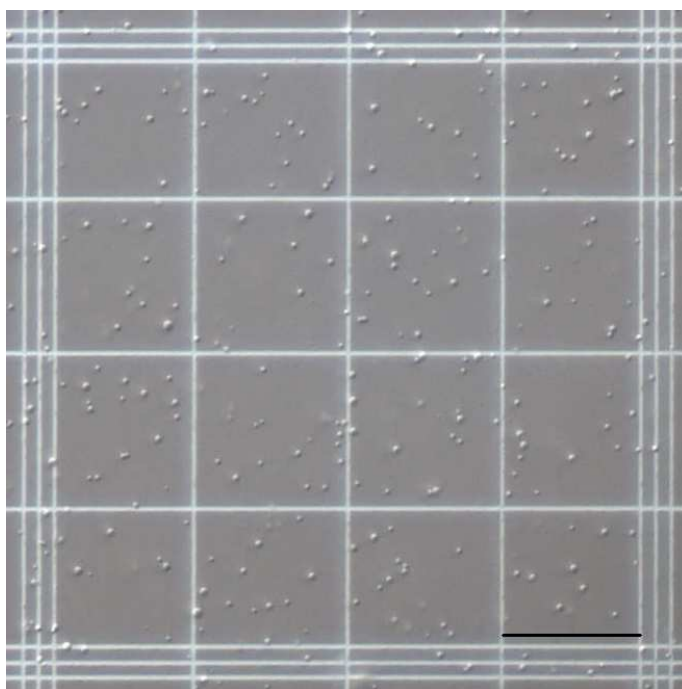


Figure 5.2. Vesicles from capsule latex seven days after flowering, collected into 250 mMol mannitol phosphate buffer. Scale bar = 50 μm .

It was evident that the largest vesicle population was present in latex sourced from the apex or capsule of the plant, at all stages of development (Figure 5.3). The GLM conducted on transformed data determined that there was a significant difference between the positions sampled ($F = 160.447$, $P < 0.001$). Capsule latex was significantly greater in vesicle number than latex sampled from both the upper stem and the stem base ($P < 0.001$ in both cases).

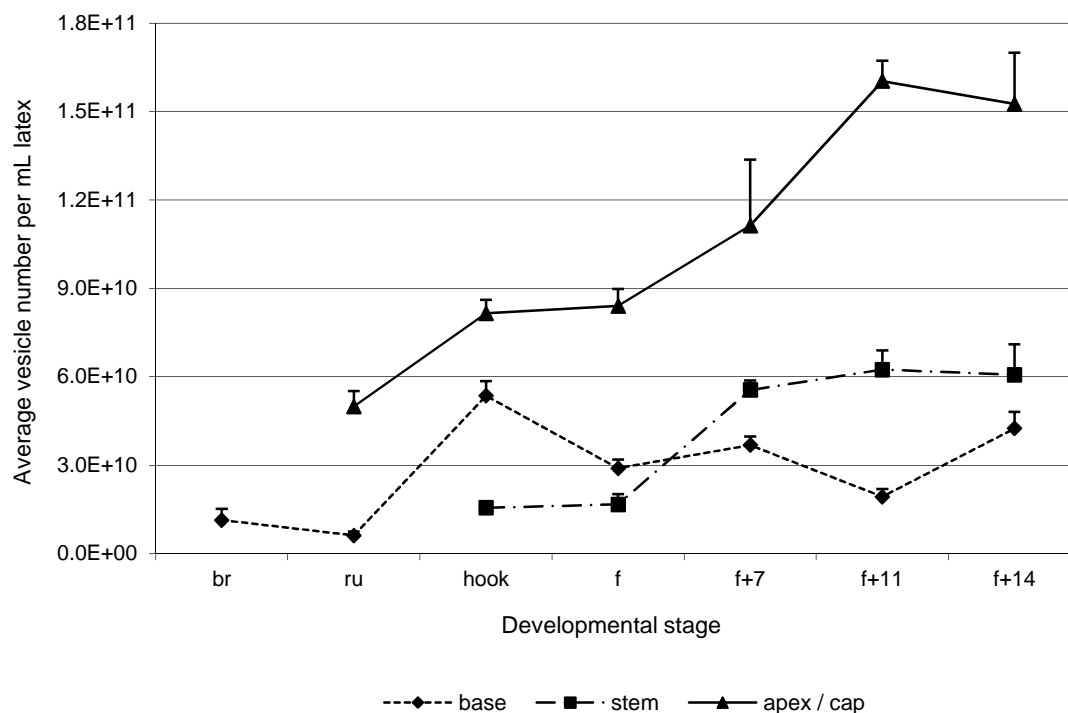


Figure 5.3. The mean vesicle number per mL latex at seven developmental stages, for *P. somniferum* latex collected into 250 mMol mannitol phosphate buffer. Latex was sampled from the base, stem and apex/capsule of the plant. Stages represented are br (basal rosette), ru (running up), hook, f (flowering), f+7 (seven days after flowering), f+11 (11 days after flowering) and f+14 (14 days after flowering). 38 plants were sampled; $n = 4$ for each of br and ru, $n = 6$ for all other stages. Upper error bars are presented, 1 SEM.

Further, a significant interaction between position and stage was detected ($F = 6.794$, $P < 0.001$), indicating that increases in latex vesicle number during development occurred differently between positions. The latex sample from the base peaked at the hook stage, but thereafter generally decreased in vesicle number and remained lower than both stem latex and capsule latex. There was a slight increase in vesicle number at f+14. In the latex of both the stem and the capsule, vesicles became more numerous during development. Vesicles in stem latex increased in number until seven days after flowering, at which point vesicle counts reached a plateau. Capsule latex vesicles also became more numerous during the developmental period studied, but vesicles accumulated at a greater rate than stem latex and for a longer duration (until eleven days

after flowering). After this stage the vesicle number appeared to decrease slightly, but there was some variability around this (evident in the SE). Sampling three positions from the same plant was not significant ($F = 1.109$, $P = 0.462$).

Note that there was little variability in the vesicle counts of base latex and stem latex, evident in the small error bars, but that the capsule latex displayed greater variability (f+7 and f+14). This indicates that vesicle numbers were relatively consistent between plants in base and upper stem latex, and that capsules were more variable.

5.4.2 *Enumeration of vesicles in latex at the capsule-stem interface*

During vesicle enumeration it became evident that vesicles were notably more numerous in latex sourced from the capsule side of the bisected thalamus than the stem side (Figure 5.4).

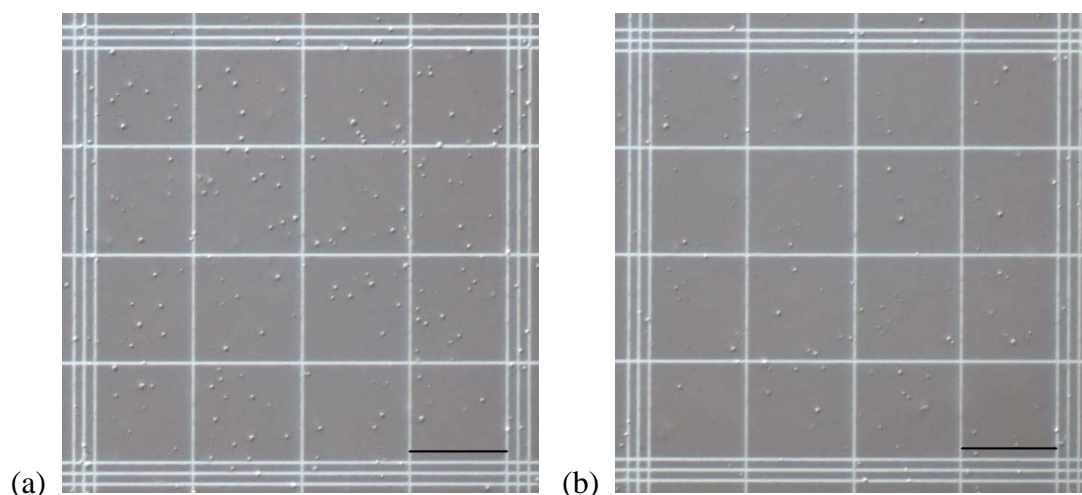


Figure 5.4. Paired images from the capsule latex (a) and stem latex (b) sourced from either side of a cut at the zone of abscission on the *P. somniferum* thalamus below the main capsule (cap_1), seven days after flowering (f+7). Each image at (a) and (b) represents one of the five haemocytometer squares counted for each latex sample. Scale bar = 50 μm .

The GLM confirmed this, revealing a significant difference in the size of the vesicle population between capsule latex and stem latex ($F = 11.985$, $P < 0.001$). Further, this was apparent for both the main axis and the first lateral branch, where capsules contained latex that was significantly greater in vesicle number than the corresponding stem latex (Figure 5.5). The average vesicle number in latex of the capsule on the main axis (cap_1) was 168% of that enumerated in stem_1 latex, and the first lateral capsule (cap_2) contained 170% of the average number of vesicles in the corresponding stem latex (stem_2).

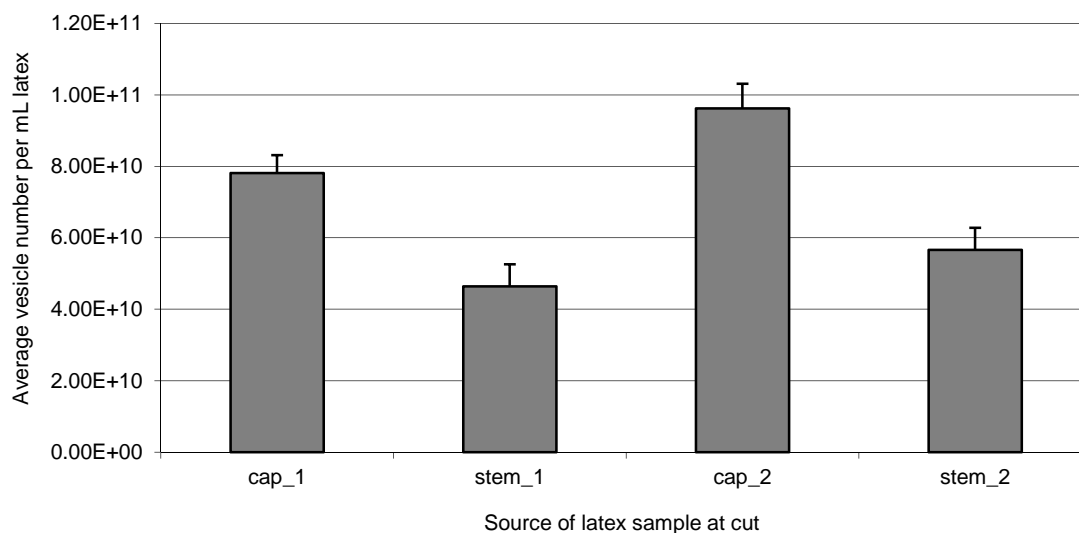


Figure 5.5. The mean vesicle number per mL of latex sourced from the apical capsule (cap_1, stem_1) and the first lateral capsule (cap_2, stem_2) of *P. somniferum* plants. Latex was sampled from either side of a single cut made at the thalamus, seven days after flowering (f+7). Six plants were sampled, $n = 24$. Upper error bars are presented, 1 SEM.

Post hoc analysis revealed that cap_1 was significantly different from stem_1 in the number of vesicles enumerated in latex ($P = 0.002$), and cap_2 was significantly different to stem_2 ($P = 0.007$). There was no significant difference in the vesicle number in latex between capsules of the main axis and first lateral branch (cap_1 versus cap_2, $P = 0.238$), nor was there a significant difference in stem latex at either position (stem_1 versus stem_2, $P = 0.696$). ‘Plant’ was not significant ($F = 0.836$, $P = 0.577$).

5.4.3 *Assessment of the volume of vesicles in latex at the capsule-stem interface*

There was a significant difference in the PVV of latex between samples sourced from capsules and from stems ($F = 11.690$, $P = 0.005$). Capsules at both stages had a greater volume of vesicles in latex (means of 43% and 47 % respectively) than in the samples taken from stems (27% and 28% respectively, Figure 5.6). Capsule latex at f+7 contained 161% the PVV of that present in stem latex, and at f+11 capsule latex contained 166% the PVV of the corresponding stem latex.

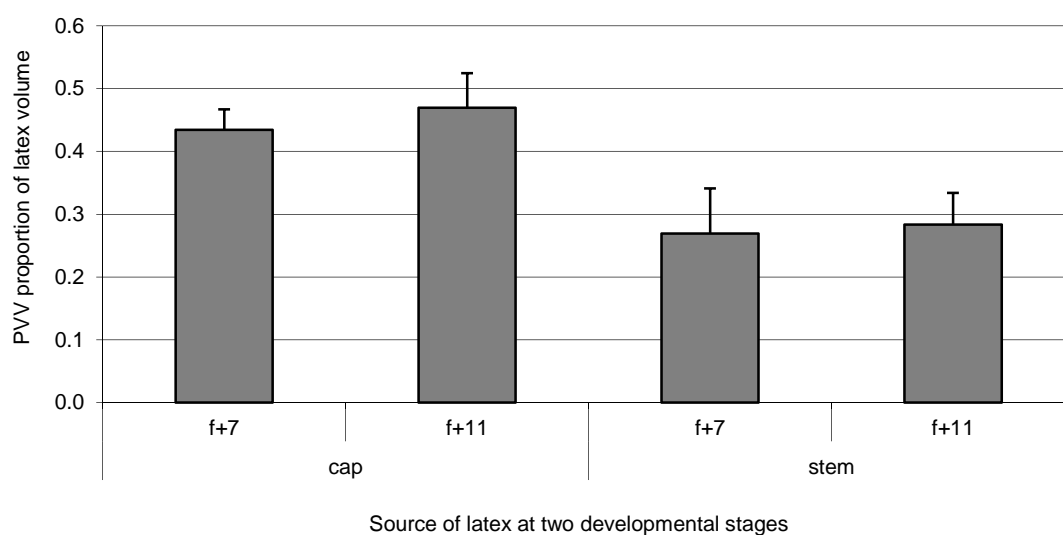


Figure 5.6. The mean packed vesicle volume (PVV) proportion of the sampled latex volume. *P. somniferum* latex samples were sourced from the capsule side and stem side of a single cut made at the thalamus. Two stages of development are represented, seven days after flowering (f+7) and eleven days after flowering (f+11), $n = 17$. Upper error bars are presented, 1 SEM.

The GLM ascertained that there was no significant difference in PVV between the two stages, f+7 and f+11 ($F = 0.251$, $P = 0.624$), although a slight increase from f+7 to f+11 in the average PVV at each position was apparent. This may not have been detected due to the sample variability, reflected in the low power of 'stage' (0.075, compared with 0.884 for 'position').

5.5 Discussion

5.5.1 Vesicle populations – number and volume

The number of vesicles in latex was significantly higher in the capsule than in samples from both the stem (10 cm below the capsule) and the stem base ($P < 0.001$, Figure 5.3). Figure 5.5 demonstrates that the significant difference between capsule latex and stem latex was also present at the top of the stem near the base of the capsule, where the thalamus was bisected at the zone of abscission, for both capsule 1 and capsule 2 (both $P < 0.001$). This significant difference in latex across a short interval suggests the presence of a very steep gradient in the vesicle number between latex of capsules and of stems in poppy plants. Further, the numbers of vesicles in latex were comparable for the two upper stem positions (Figure 5.3, and Figure 5.5), indicating that latex is relatively consistent in vesicle number along the stem axis. It is apparent then, that in terms of vesicle number, capsule latex and stem latex are distinctly different, and the disparity occurs across the thalamus region beneath the capsule.

The difference in latex vesicle number is concomitant with differences in vesicle volume. Both the number of vesicles and the volume of vesicles in capsule latex were significantly different from that present in stem latex. PVV in latex was significantly higher in capsules than stems (Figure 5.6, $P = 0.005$), with capsule latex containing 161% the PVV of that present in stem latex (at f+7). The proportion is consistent with the enumeration investigation of (c), where vesicle numbers in the capsule latex were 168% of those determined in the stem (Figure 5.5, at f+7).

Whilst it is apparent that vesicle size was variable (Figures 5.2 and 5.4), a feature that has been noted by other authors (Decker *et al.* 2000, Thureson-Klein 1970), variability in size was evident in vesicles of both capsule and stem latex. Thus, based on the significant difference in both vesicle number and PVV between capsule and stem latex, and the congruence of proportions for vesicles numbers and PVV, it was concluded that vesicle number was the main contributor to increases in PVV in latex.

Pham and Roberts (1991) identified increases in ‘vacuolar volume’ over the three-week period following flowering, but could not identify whether vesicle volume related to differences in size or number. Although not directly comparable to these results (as these authors pooled latex from capsule and stem and there is some ambiguity in the methods regarding vacuolar volume), a consideration of the steep upward trend in vesicle number apparent in Figure 5.3 of this investigation suggests that an increase in vesicle number may offer an explanation for the increases in vacuolar volume revealed in their study – at least until f+11. Beyond this time vesicle number appeared to plateau (Figure 5.3) and no data was collected after f+14, but it is plausible that the further increases in volume observed by Pham and Roberts (1991) later in development may have been attributable to the commencement of capsule desiccation. Water relations in the capsule do not remain static over time; both diurnal changes in latex (Itenov *et al.* 1999) and capsule developmental changes have been noted, especially after f+14 (Bunting 1963, Prokof'ev *et al.* 1981, Prokof'ev *et al.* 1983) so it cannot be assumed that latex will be consistent in water content.

5.5.2 Vesicle populations – development

The observed steep gradient in both the PVV and the number of vesicles at the zone of abscission on the thalamus (of both cap_1 and cap_2) suggest that capsule latex and stem latex are developmentally different. This was evident in the vesicle development study (section 5.4.1), where the three positions demonstrated different trends in the accumulation of vesicles over seven stages of development (Figure 5.3), and this was borne out in the significant interaction of position and stage ($P < 0.001$).

At hook stage (the first stage that assessed all three positions) there existed a significant difference in baseline vesicle numbers per millilitre of latex in samples taken from the bud and the stem (5x greater in the bud). Nessler and Mahlberg (1977b) indicated that perforations in both lateral and transverse walls of laticifers usually occur late in differentiation, by which time vesicles are plentiful within the cell. This would have negative ramifications for the bulk movement of latex from the stem into the bud, as latex would be moving against an established concentration gradient of vesicles.

After flowering both positions increased latex vesicle number, although vesicles in the capsule latex accumulated at a greater rate and for a longer duration than latex sampled from the stem. For the two lower positions, the pattern of vesicle accumulation during the period studied was consistent between replicates (evident in the small errors), yet capsules demonstrated greater variability, perhaps as a result of the rapid development of the fruit (whereas stem development had previously occurred).

Flowering was the pivotal developmental stage for latex vesicle number. This was evident in the presence of a sharp increase in latex vesicle number for both capsule latex and stem latex in the week after flowering. This coincides with the previously observed

pattern of rapid alkaloid accumulation during the week after flowering (Huang and Kutchan 2000, Roberts *et al.* 1983), and it concurs with the development of laticifer volume as explained in section 2.5.

Laticifer volume, or latex capacity, was defined as the proportion of the capsule wall allocated to laticifers. It was evident that latex capacity was fixed relatively early in development, before the first week after flowering, as capacity had reached a maximum at sample stage 1, f+7 (section 2.5, Figure 2.2). Bernáth (1989) demonstrated that one third of capsule dry mass had developed by flowering stage, and it was apparent that the laticifer system was also extensively developed (per unit tissue) at one week after flowering. Thus, during the days after flowering, much of the cellular ‘infrastructure’ is present in capsules, the sub-cellular machinery is available for vesicle production (ER), the latex quickly fills with vesicles (Figure 5.3), and with storage vesicles in place (and continually being produced), rapid alkaloid accumulation occurs (Decker *et al.* 2000, Huang and Kutchan 2000, Nikonov 1958, Roberts *et al.* 1983). As significant differences in vesicle number were noted between the capsules and stems at two positions in this investigation, this process is probably closely related to, and regulated by, fruit development.

The developmental regulation of alkaloid biosynthesis has been described by several authors (Facchini and Bird 1998, Facchini and Park 2003, Huang and Kutchan 2000, Kamo and Mahlberg 1984) and indeed, there may be a relationship between the vesicle population and alkaloid content, regulated by developmental cues. The rapid increase in latex vesicle number during development (Figure 5.3) suggests that there may be a relationship between vesicle number and alkaloid yield, further to the work of Pham and

Roberts (1991) who identified a relationship between the vacuolar volume of 900 xg and 1100 xg components, and alkaloid yield. Although the current investigation did not fractionate the vesicle component, Decker *et al.* (2000) in their study of fractionated capsule latex found the alkaloid-protein ratio increased with plant development for all of the separated vesicle fractions, thus it could be expected that such a relationship exists. Additional investigations with flow cytometry may elucidate these relationships.

5.5.3 Differences between capsule and stem latex at the capsule-pedicel junction

It becomes apparent then, that the vesicle population in capsule latex is distinct from that of stem latex – they are significantly different in vesicle number and PVV, capsule latex has a higher baseline vesicle number, and the two positions exhibit different patterns of vesicle accumulation, all occurring at the zone of abscission on the thalamus. There are further indications of a distinction in latex at this location through the work of Kamo and Mahlberg (1984). These authors sampled latex from the capsule, the capsule-pedicel junction, and the lower pedicel to ascertain the capacity for biosynthetic activity. Using the supernatant of centrifuged latex samples, they found that capsule-pedicel latex had the greatest capacity for conversion of ^{14}C -dopa into dopamine, followed by capsule latex and then lower pedicel latex. Also, the capsule latex had the highest protein content, containing 130 mg/mL supernatant versus 42 mg/mL for that of the capsule-pedicel latex. Whilst it could be argued that centrifugation may have disrupted fragile vesicles and released proteins and/or enzymes (Griffing and Nessler 1989, Kutchan *et al.* 1986, Roberts and Antoun 1978), the subcellular location of these is not at issue. The latex of all positions experienced the same experimental conditions, and it is the presence of a significant gradient that is the crucial result. Kamo and Mahlberg (1984) identified differences between the capsule latex and capsule-pedicel

latex in biosynthetic capability, and discerned a large gradient in the protein content. These factors constitute another two characteristic differences in poppy latex at this location beneath the capsule.

Very early research had also implicated the presence of a distinction between capsule and thalamus latex in terms of metabolic capabilities (Fairbairn and Djoté 1970), however it is difficult to credit their observations due to the lack of clarity in the published work and the variability in incorporation of labelled substances (not uncommon in radio-labelling experiments, Poeaknapo *et al.* 2004). More recent investigations however, such as that of Bajpai *et al.* (2001), do lend credence to the idea that there are dissimilarities in biosynthetic function between the pedicel and capsule. These authors found no relationship between the ratios of codeine to morphine in the pedicel versus that in the capsule, and cited the lack of congruence to be a result of differential alkaloid synthesis in these structures.

Thus, due to the differences noted between capsule and stem latex (across the thalamus) from this study, and the findings of other researchers (Bajpai *et al.* 2001, Kamo and Mahlberg 1984) which support the notion that capsule latex is distinct from that of stem latex, it is proposed that *in situ* production of vesicles is the main source of increase in latex vesicle number. Also, the variability in vesicle size (approximately 2 μm) observed at each stage, and in both capsule and stem, may be further evidence for *in situ* production if a relationship exists between size and development. Indeed, it may be that the larger vesicles represent the ‘heavier’ fraction of latex, which Decker *et al.* (2000) posited as being more developed than the lighter fraction.

Thus, the proposed *in situ* production of vesicles begins to cast doubt on the translocation of alkaloids to the capsules via vesicles through the bulk movement of latex; and therefore the occurrence of latex movement *in toto* as suggested by Fairbairn *et al.* (1974) must be questioned.

5.5.4 Latex translocation – *in toto*

In several studies, Fairbairn and El-Masry (1967) and Fairbairn *et al.* (1964, 1974) supplied labelled morphine to peeled stems of poppy plants, and were able to detect the presence of (varying) levels in the capsule tissue and latex. Their *in vivo* work demonstrated that rapid movement of radio-labelled morphine occurred from stem to capsule, the authors reporting rates of translocation in the range of 5 cm to 7.5 cm per hour in one of their studies. The authors then proposed that the translocation of vesicles occurred ‘partly by an *in toto* upward movement of the latex’, but did not address issues of replication and questionable methodology. It would be expected that if there was bulk movement of latex from stem to capsule, especially at the rates of movement described by Fairbairn *et al.* (1974), that there would be a gradual change in latex characteristics (such as vesicle number and PVV) and, that there would be some congruence in functional characteristics (such as biosynthetic and/or metabolic capability). It is apparent that this is not the case.

The more plausible explanation is that latex vesicles are produced *in situ* during development and that alkaloids are sequestered into vesicles thereafter, but that *in toto* movement of latex does not occur. This accounts for differences in vesicle number, PVV, different patterns of development, and is consistent with previous research that identifies other disparities between capsule and stem latex (Bajpai *et al.* 2001, Kamo

and Mahlberg 1984). Further, the numerical increase of vesicles in capsule latex is very rapid, yet the lower positions are very stable and consistent in number. During the dramatic population increase in capsule latex from flowering to f+11, an average of 6.94×10^9 vesicles (for every millilitre of latex present in the capsule) would have to be transported into the capsule per day, not accounting for *in situ* production. Even considering capsule vesicle production, latex from the stem is not as vesicle-dense as the capsule, and the cytosolic portion would dilute the capsule latex. To increase the vesicle number in capsule latex through bulk flow would require a considerable volume of stem latex to be moved rapidly into the capsule, and concentration of capsule latex would also need to occur. It may be posited that root pressure could provide the impetus for the upward movement of latex, and that the concentration of capsule latex may be achieved through water loss via the copious stomata in the capsule epidermis and in the endocarp (Kapoor 1995), and indeed there is evidence that diurnal fluxes in water and alkaloid content do occur in latex (Itenov *et al.* 1999, Shukla *et al.* 1996, Yadav *et al.* 2004). However, the steep gradient in latex characteristics at the thalamus is yet to be explained.

A further consideration for bulk flow is the anatomy of the laticifer system through the stem, pedicel, thalamus and capsule. Both the stem and pedicel are similar in their anatomical structure, however the pedicel has fewer vascular bundles (Petri and Mihalik 1998), and the microscopical investigations of Fairbairn and Kapoor (1960) describe an intricate course of vascular bundles (and laticifers) once they arrive at the thalamus. In the thalamus both the direction and connection of vascular bundles is altered, giving rise to many trace bundles that accommodate the supply to floral structures such as the sepals, petals and stamens – all of which contain latex (Fairbairn and Kapoor 1960, El

Kheir 1975, Nessler and Mahlberg 1976). Indeed, it may be hypothesised that through the abscission of floral structures many of these laticifers may become occluded with callose, present in older laticifers of poppy (Nessler 1976 in Nessler and Mahlberg 1977b), and in a process similar to that observed in laticifers of the petioles of *Hevea brasiliensis* (Spencer 1939a). It is unlikely however, that if this did occur it would have contributed to the gradient of vesicles noted between capsule and stem latex, as the disparity in vesicle number exists at hook and flowering stages, prior to floral parts being abscised. The idea to be considered, however, is the impact of the anatomy at this point in the thalamus, and whether it could possibly constitute a bottleneck in the suggested upward pathway of latex to capsules.

Therefore, based on the results of this study and prior research, it appears that bulk latex movement is unlikely. The more parsimonious explanation of *in situ* vesicle production in the generation of a steep vesicle gradient, and the other cited evidence against bulk latex flow emphasises the improbability of the suggestion by Fairbairn *et al.* (1974). It is doubtful that movement of alkaloids via the vesicles is effected by *in toto*, upward movement of latex.

5.5.5 Translocation - vesicle trafficking into the capsule

Although it appears that bulk latex movement from stem to capsule may be unlikely, the possibility of the movement of vesicles within latex should at least be addressed. Vesicles have been demonstrated to perform a role in the intracellular trafficking of a range of plant substances (Grotewold *et al.* 2004, Snyder and Nicholson 1990), including the alkaloid, berberine, in *Coptis japonica* (Otani *et al.* 2005). It may be proposed that the gradient observed across the thalamus in *P. somniferum* is generated

by the trafficking of transport vesicles through the articulated system of laticifers. For this to occur however, stem latex would be required to rapidly supply and traffic a large number of vesicles against a concentration gradient as indicated above. The hypothesised process would require an energy-dependent mechanism to actively concentrate vesicles in the capsule, and considering the difference between stems and capsules in alkaloid content and concentration (Bajpai *et al.* 2001), the heterogeneity of vesicles in their content (Alcantara *et al.* 2005, Kutchan *et al.* 1986), and with the recognition that several cell types (other than laticifers) are involved in the synthesis of alkaloids (Bird *et al.* 2003, Samanani *et al.* 2006, Weid *et al.* 2004), it is doubtful that upward relocation of vesicles containing alkaloids would occur in latex.

5.6 Conclusions and recommendations

This investigation established that capsule latex was significantly greater in vesicle number and vesicle volume (PVV) than stem latex, and that this difference occurred as a steep gradient around the capsule-pedicel junction, on the thalamus of the poppy capsule. It also demonstrated that latex vesicles increased in number during plant development, and that the pattern of development varied, dependent on position or location in the plant.

The study also addressed the likelihood of bulk flow or *in toto* movement of latex, and concluded; that due to the disparity in vesicle populations between capsule and stem, the steep gradient over which this occurred, the different patterns of development of the vesicle populations, and the evidence from other studies that demonstrated characteristic differences in the latex at the capsule-pedicel junction (both biosynthetic activity and protein content); that the occurrence of bulk flow or *in toto* movement (as suggested by Fairbairn *et al.* 1974) is improbable.

Further to this, several lines of investigation are recommended:

- Further elucidation of the capsule and stem vesicle populations. Whilst this study quantified the populations in capsule and stem latex, it did not characterise the subpopulations in each. Flow cytometry is a technique that can (for vesicles) quickly enumerate, size, characterise subpopulations and identify vesicle contents (Lingua *et al.* 1999, Mérigout *et al.* 2002, Murphy 1985, Schröder and Petit 1992), and as such would be vital in defining the nature of the capsule and

stem latex vesicles. It could provide further insight into the storage and biosynthetic capacity of the latex of different organs, and identify relationships with alkaloid yield, avoiding issues associated with sucrose density fractionation. Further, as a rapid technique with high through-put, it would be possible to process sufficient samples to generate a regression model to assess whether the population of vesicles in the stem latex could predict that of capsule latex.

- The influence of developmental cues and hormonal influences on the vesicle population in laticifers merits investigation, especially during the period from bud development until seven days after flowering.
- The presence of a vesicle gradient warrants further inspection, and a study of the thalamus region from the period of bud development onward, using current microscopical techniques, would be useful.
- It may be that the presence of a gradient is not the result of a physical barrier. With *in situ* vesicle production it could be that pressure differentials within the laticifer system aid in the maintenance of the population in the capsule. Development of techniques to assess pressure would be valuable.

CHAPTER 6 The osmotic potential of poppy latex and the testing of a pressure probe for the assessment of capsule and stem exudation pressure in *Papaver somniferum* L.

6.1 Introduction

Although the bulk movement of latex from stem to capsule in *Papaver somniferum* L. appears unlikely, further evidence is required before it can be discounted, as too little is understood of laticifer physiology (Pickard 2008). It is postulated that if bulk movement occurs, a possible mechanism for translocation could be that of water-driven pressure differentials, moving latex from lower in the plant to the rapidly growing and transpiring capsules (Prokof'ev *et al.* 1984), akin to the movement of solutes in phloem (Daudet *et al.* 2002, Gould *et al.* 2005, Hölttä *et al.* 2006). The increasing concentration of capsule latex components would then result from water loss via the plentiful stomata in the capsule epidermis (Kapoor 1995), at least until 12 days after flowering, when transpiration decreases (Prokof'ev *et al.* 1984). If this process occurs, it may be identified through patterns of variation in latex osmotic potential and turgor pressure.

The latex of *P. somniferum* is not static – daily variations are evident in alkaloid concentration (Shukla *et al.* 1996) and water content (Itenov *et al.* 1999). Indeed, the water content of poppy latex has been reported to vary from 31% (Yadav *et al.* 2004) to 88% (Baier *et al.* 1992), with Itenov *et al.* (1999) illustrating a diurnal variation of approximately 10% (65% to 75% water). Thus, it would be reasonable to expect differences in osmotic potentials and turgor pressures within laticifers, and that these

fluxes may provide a mechanism by which bulk latex movement may occur from stem to capsule.

Direct research into these parameters in poppy appears scant. Prokof'ev *et al.* (1981), and Prokof'ev *et al.* (1983) investigated water relations in the ripening capsules and observed that osmotic pressures in placenta increased during capsule development but in seeds they decreased, suggesting the desiccation of seeds by loss of water to the placenta. These authors went on to illustrate that the differential allocation of osmotically active substances (such as potassium ions and sugar) influenced water potential gradients, but their results pertained to whole tissue, not extracted latex.

Osmotic potential has been studied for other latex-bearing species such as *Hevea brasiliensis* (Milburn and Ranasinghe 1996) and *Musa* sp. (Baker *et al.* 1990, Milburn *et al.* 1990). For *H. brasiliensis*, the osmotic potential of latex was recorded as approximately -1.0 MPa, with a notable decrease during the middle of the day (Milburn and Ranasinghe 1996). These authors also described the manometric pressure potential of latex, and found that it peaked at 1.3 MPa in the morning and declined to 0.5 MPa at midday. For *Musa* sp., the recorded osmotic potential was less negative, ranging from -0.036 to -0.70 MPa, with diurnal variations apparent (Baker *et al.* 1990) and with the most negative potential apparent in the initial samples taken. Milburn *et al.* (1990) recorded pressures of $0.25 - 0.35$ MPa for *Musa* sp. latex turgor pressure. There are no apparent reports of latex osmotic potential or exudation pressures for poppy.

The intent of this chapter was to conduct a pilot study to direct future research, and as such, the aims of this investigation were to:

1. assess the osmotic potential of latex in the capsule and stem of poppies
2. test methods for the assessment of latex exudation pressure in poppies.

6.2 Materials and methods

6.2.1 Growing conditions

Bubble manometers were tested in the field, in commercial *P. somniferum* crops located in the Coal River Valley (southern Tasmania) during the southern hemisphere summer of 2005/2006. Details of the field trials are outlined in Chapter 2, Materials and methods (section 2.3.1). Plants used for the development of a pressure probe and in the investigation of osmotic potential were grown under controlled glasshouse conditions as described in Chapter 5, Materials and methods (section 5.3.1).

6.2.2 Sampling methods

6.2.2.1 Method development – field pressure testing with bubble manometers

Bubble manometers were devised by inserting a flame-sealed glass capillary tube into a plastic pipette tip (after Milburn and Ranasinghe 1996). The plastic pipette tip, with a smaller diameter than the capillary tube, was used to aid insertion and ensured that a plug of capsule tissue did not block the capillary tube when the manometer was inserted into the capsule exocarp. The tip also acted as a protective collar and prevented snapping of the capillary tube (Figure 6.1).



Figure 6.1. Diagrammatic representation of a bubble manometer devised to test the exudation pressure of latex in the capsules of *P. somniferum* L.

To secure the capillary tube in the plastic tip and seal the junction of plastic and glass, it was embedded in an epoxy-resin (Araldite®). Trials were conducted with several types of tip – a standard production flat tip, a standard production bevelled tip and a tip that had been cut on a 45° angle.

It was found that the exodermis of poppy capsules (including the cuticle, epidermis and hypodermis; Kapoor 1995) was difficult to penetrate easily. The capsule exodermis was very robust, and the application of sufficient pressure to pierce the capsule often punctured the pericarp entirely, allowing latex to escape to the capsule interior rather than filling the capillary tube. On the occasions that piercing was successful the method worked effectively and latex rapidly filled $\frac{1}{2}$ to $\frac{3}{4}$ of the capillary tube, however, although portable and convenient for testing field grown plants, the constructed bubble manometers generally lacked precision and repeatability (also observed by Milburn *et al.* 1990). Thus, an alternative method was developed to test hydrostatic pressure in an attempt to achieve greater control, precision and accuracy.

6.2.2.2 *Method development – laboratory testing with customised pressure probe*

A customised pressure probe technique was developed to determine the exudation pressure of laticifers in poppy capsules and stems (after Tomos 2000, Tomos and Leigh 1999). The probe was a disposable, 18-gauge (18G) Terumo surgical steel syringe needle (38mm) with 1.2mm diameter (needles with smaller diameters were tested but they did not seal well and often would not remain within the sample). The probe was connected to plastic tubing that was completely filled with canola oil (via the plastic tap), excluding all air (Figure 6.2). A 5psi pressure transducer (Omega PX26-005GV, with 4 pin connector, CX136-4) detected the compression of oil resulting from the

positive hydrostatic pressure of latex. The probe was manually inserted into capsules (or stems) of intact, potted *P. somniferum* plants at the f+7 developmental stage. Capsules and stems were tightly wrapped in Parafilm™ to prevent leakage of latex upon insertion of the probe. No plug of tissue was detected inside the needle and it appeared to pierce the tissue easily (unlike the bubble manometers).

Exudation pressure readings were taken from capsules at a point on one of the depressions in line with stigmatic rays (coincident with vascular bundles and therefore laticifers) and from the stem.

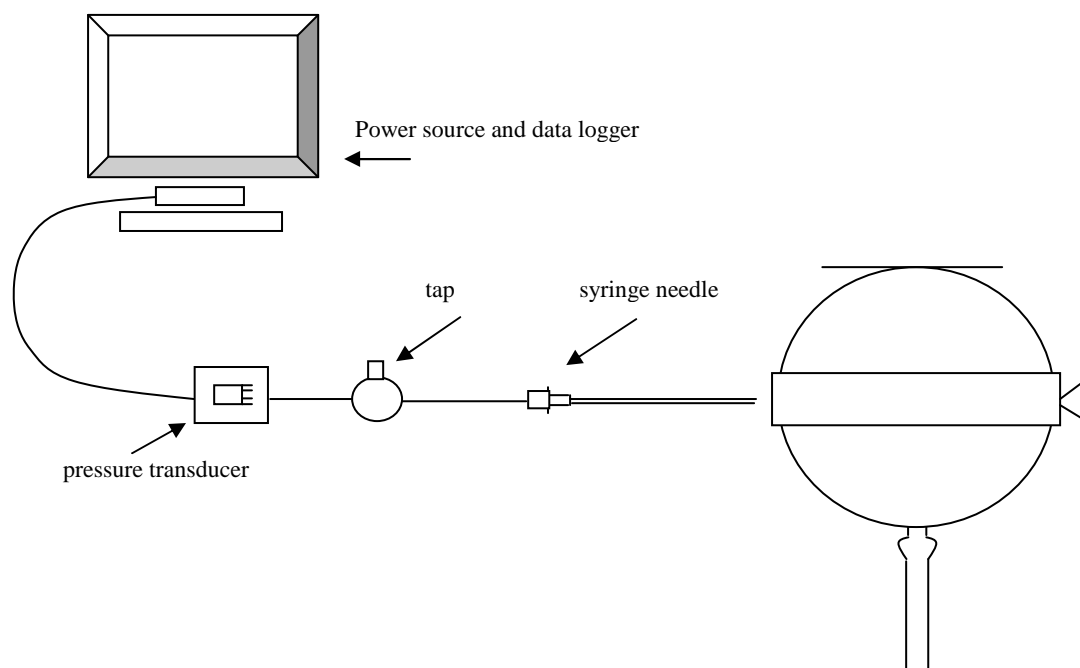


Figure 6.2. Diagrammatic representation of a pressure probe developed for the assessment of exudation pressure of latex in *P. somniferum* capsules and stems. An 18-gauge surgical steel syringe needle was attached to a piece of tubing filled (via the tap) with canola oil. A 5psi pressure transducer was used for pressure detection; binding the capsule in Parafilm™ facilitated insertion.

6.2.2.3 *Assessment of osmotic potential in latex of capsules and stems*

Measurement of latex osmolality

The osmolality of latex sourced from ten *P. somniferum* plants was investigated at several locations on each plant, when the main capsule (cap_1) was at stage f+3 (3 days after flowering). Samples were extracted early in the morning to avoid diurnal fluctuations in water content (Itenov *et al.* 1999), from the capsule, from the stem just below the capsule thalamus (from the pedicel), and from the base of the stem. The main axis (cap_1) and first lateral branch (cap_2) were sampled. Samples were collected by pipette from the top of the capsule after the removal of several stigmatic rays, and from the cut surfaces at the upper and lower stem positions (Figure 6.3).

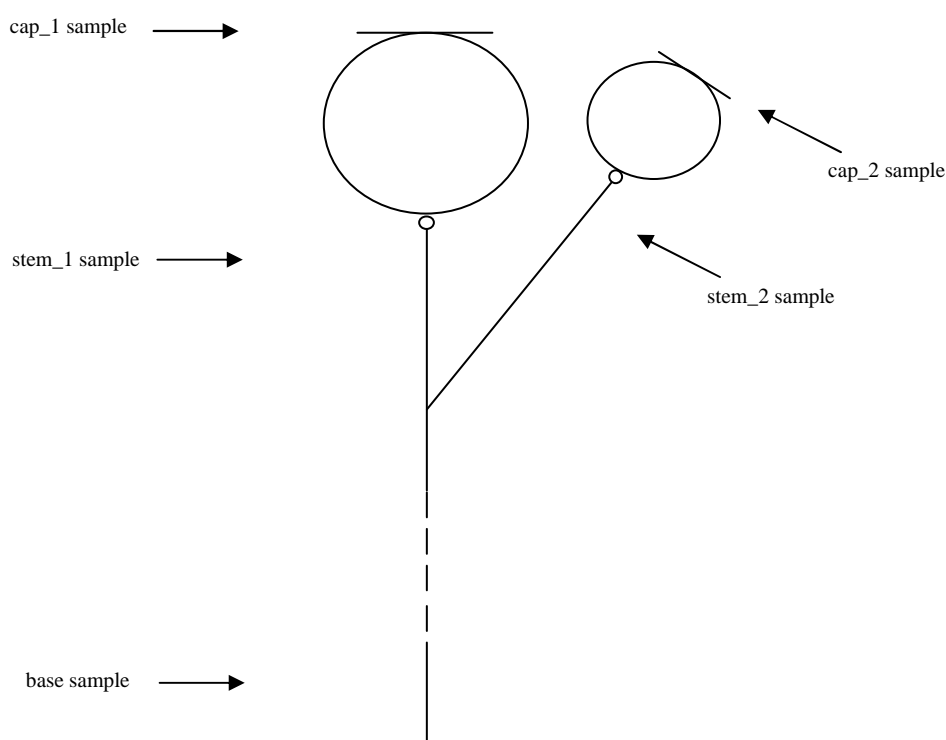


Figure 6.3. Diagrammatic representation of a *P. somniferum* plant, with latex sampling positions indicated by arrows. Samples were collected by pipette after sections were made at indicated locations. Sampling was destructive.

The collected latex was pipetted into labelled 1 mL Eppendorf tubes and kept on ice until analysed (within one hour) using a calibrated Vapro® vapor pressure osmometer, Wescor model number 5520. A 10 μL sample was extracted from each Eppendorf using a Drummond Wiretrol®1 micropipettor and placed on a paper disc in the osmometer chamber. Readings were taken after a 3-minute period of equilibration. Units of osmolality (mosmol kg^{-1}) were converted to osmotic potential (ψ_s) to allow for comparison with other *P. somniferum* literature.

6.2.3 Data analysis

Assessment of osmotic pressure in latex of capsules and stems

The data were assessed for normality by an Anderson-Darling test (A-squared = 0.481, $P = 0.220$, $n = 41$) and variances were stable (Levene's test; $P = 0.861$). Analysis was undertaken by using a GLM in SPSS v17.0, with 'position' as a fixed factor and 'plant' as a random factor, accounting for multiple sampling of one plant. Tukey HSD was employed for *post hoc* analysis.

6.3 Results

6.3.1 Method development – testing of exudation pressure

The exudation pressure detected in capsule was $1.78 \text{ KPa} \pm 0.39$ (mean, SE) and in the stem it was $0.99 \text{ KPa} \pm 0.23$. Although the average capsule pressure was higher than that of the stem and the highest pressure overall was observed in the capsule, it should be noted that identification of the position of vascular bundles is more reliable in the capsule. Additionally, pressure readings were variable (especially those of the capsule) and experimental power for the trial was poor (0.426).

Further, recorded pressures were very low. This may be partly explained by the fact that testing was performed in the afternoon, and that these values represent broader pressures, not individual laticifer pressures. However, the observed low pressures may be a result of internal tissue damage by the probe causing undetected leakage, and thus experimental inaccuracies cannot be discounted. A microcapillary pressure probe used directly on laticifers (with capsule exodermis removed) may be an advance in terms of technique.

Therefore, the combination of variability and lower than expected pressure readings indicates that this method requires further validation and optimisation, however comprehensive method development was beyond the scope of this study. As a result it would be inadvisable to speculate on the trial results, and therefore they will not be addressed in the discussion.

6.3.2 Assessment of osmotic pressure in latex of capsules and stems

There was a significant difference in the osmotic potential of latex sourced from several positions on the plant ($F = 18.929$, $P < 0.001$), but there was no effect of removing several samples from one plant ($F = 1.670$, $P = 0.146$). Mean osmotic potential in latex ranged from -0.72 MPa in the base to -1.02 MPa in capsule 2, with capsules displaying greater osmotic potential than the base and stem_1 (Figure 6.4).

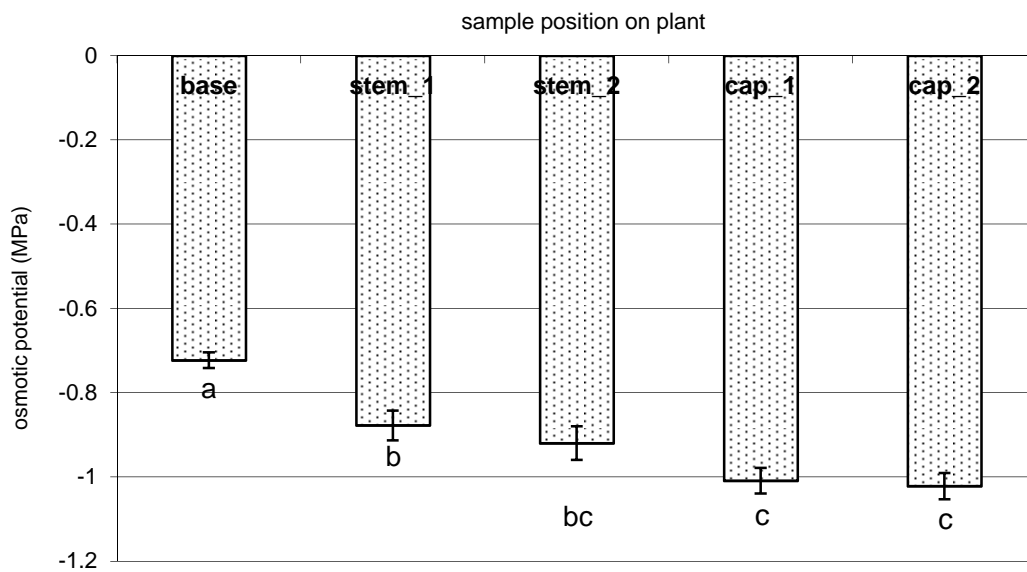


Figure 6.4: The mean osmotic potential of latex (MPa) sampled from several positions on poppy plants; the base of the stem, from the upper stems of capsules 1 and 2, and from both capsules. Upper error bars are presented, 1 SEM.

Post hoc analysis revealed that the osmotic potential of latex from the base was significantly different from that of all other positions (from stem_1, $P = 0.006$; stem_2, $P = 0.001$, and both capsules $P < 0.001$). The osmotic potential of the main capsule (cap_1) was significantly different from that of the corresponding stem latex (stem_1, $P = 0.033$) but it was not significantly different from that of the first lateral capsule

(cap_2, $P = 0.999$). Cap_2 osmotic potential was also significantly different from stem_1 osmotic potential ($P = 0.021$) but not from stem 2 osmotic potential ($P = 0.239$).

6.4 Discussion

This study revealed plant-level differences in the osmotic potential of poppy latex at stage f+3 (of the main capsule, cap_1), with the mean osmotic potential ranging from -0.72 MPa in the base to approximately -1.0 MPa in the capsules. This is comparable with other studies of osmotic potential in poppy capsules (Prokof'ev *et al.* 1981 and Prokof'ev *et al.* 1983). The osmotic potential was significantly more negative in the capsules than in both stem_1 (the main stem) and the base of the main stem (Figure 6.4), demonstrating that in the early stages of development the capsule latex has more osmotically active solutes than latex from other positions. This is consistent with; fruit sink strength, especially of the upper two capsules (as discussed in Chapter 3, section 3.5.2); with previously observed differences in latex contents of capsule and stem (Chapter 5, section 5.5.3); and also in terms of the turgor requirements for cell expansion (Daudet *et al.* 2002, Pritchard 1994).

The finding that capsule latex contains more osmotically active solutes than stem latex at f+3, is also consistent with laticifer cell development. Turgor is dependent on local water potential (Daudet *et al.* 2002) and solute concentration, therefore the more negative osmotic potential in the capsule latex (created by high solute load) would cause an influx of water from nearby tissues, increasing cell turgor (Hölttä *et al.* 2006). Indeed, water fluxes between laticifers and nearby cells have been identified in other species (Buttery and Boatman 1966, Tungngoen *et al.* 2009), and Itenov *et al.* (1999)

identified the occurrence of water fluxes for poppy laticifers. Turgor pressure is integral to cell growth (Lockhart 1965, Pritchard 1994) and it was demonstrated in Chapter 2 (section 2.4) that laticifers attain per-unit volume early in capsule development (before f+7) and maintain volume thereafter. Thus, laticifer cells would have an increased turgor requirement during capsule development (stem laticifers already developed) in order to achieve capacity (per unit) prior to f+7, and to then keep pace with the rest of the capsule as it rapidly expands. Tomar *et al.* (1990) demonstrated that water stress imposed at the bud and early capsule stages of *P. somniferum* reduced subsequent husk yields of more than 30%, and other researchers have also discussed the importance of timing and quantity in irrigation (Chung 1987, Szabo *et al.* 2008); illustrating the importance of turgor in cell expansion at this stage for poppy.

Similar patterns of the development of laticifer volume have been described for other organs of latex-bearing species, for example, the leaves of *Euphorbia pulcherrima*, yet these are non-articulated laticifers and have a different mode of growth (Spilatro and Mahlberg 1986). Turgor pressure not only satisfies the developmental requirements of these cells; it also enables the laticifers to perform their role in defence against predation through copious exudation upon injury (Agrawal and Konno 2009, Fahn 1989, Hagel *et al.* 2008).

Latex is biochemically complex and contains osmotically active solutes including ions, a variety of proteins and salts (Decker *et al.* 2000, Nessler *et al.* 1985, Pham and Roberts 1991, Shukla and Krishna Murti 1971), but the work of Baier *et al.* (1992) and Gould *et al.* (2004) indicates that a predominance of potassium ions in latex may be a major contributor to osmotic potential. Prokof'ev *et al.* (1981) and Prokof'ev *et al.*

(1983) also linked osmotic potential to potassium ions and sugar content for poppy capsules, however they used whole tissue in their determinations and did not analyse fresh latex. Beyond this there is little direct research into the osmotic constituents and water relations of capsule laticifers in poppy, so there is no definitive description of osmotically active solutes and their role in the generation and maintenance of laticifer turgor. There has also been a lack of research into the possibility of solute loading from the surrounding cells, noted to occur for laticifers in other species (Fay *et al.* 1989, Inamdar *et al.* 1988, Zhang and Ma 1995) and with the observation of plasmodesmatal connections between laticifers and sieve cells by Facchini *et al.* (2007), it is a possibility that warrants examination.

Although the observation of significantly higher capsule osmolality appears consistent with capsule physiology and development, possible sources of experimental uncertainty should be addressed, especially in light of the lack of research in this area. It may be suggested that greater osmolality in the capsule latex might have arisen through contamination by phloem sap, possible as laticifers are located adjacent to sieve cells (Facchini and Bird 1998, Fairbairn and Kapoor 1960) and both would rupture at the wound site (Baker *et al.* 1990). Phloem cells rapidly become occluded upon rupturing (van Bel 2003, Eschrich 1975), and with the copious exudation that occurs from laticifers it may be posited that phloem contamination would represent a small volume of the collected sample. Whilst this hypothesis is probably valid, further investigation of the osmotic contents of *P. somniferum* latex would be advisable in order to elucidate the contribution of phloem contamination in such contexts.

It would also be advisable to assess changes in exuded latex content over time. Latex was collected into sealed tubes and held on ice to avoid evaporation and water loss, but there was a lag time after collection and samples were left in the pressure chamber of the osmometer for three minutes to equilibrate. There may have been dissociative or precipitative processes occurring within the latex once exuded (Facchini and Bird 1998), which may have altered the number of osmotically active solutes. A study of processes within exuded latex would further elucidate osmotica and latex biochemistry, and would inform on coagulation systems (De Gussem *et al.* 2006).

A further consideration of the observed differences in osmotic potential is the consequence this would have in terms of hydrostatic pressure within laticifers. It may be expected that if capsule latex has a greater concentration of osmotically active solutes, osmotic potential would be more negative and capsule laticifers may have greater turgor pressure than stems. Techniques, including the use of a microcapillary pressure probe to investigate individual cells, need to be further pursued to examine the water relations of laticifers.

The detection of differences in the osmotic potential of poppy latex has revealed an under-investigated yet very important aspect of laticifer development and physiology, and has raised a number of questions. For example - how consistent is osmotic potential during development? What happens in laticifers after latex exudes upon puncturing and cell volume decreases? How does refilling of vessels occur? Are there mechanisms of loading and water flow into the laticifer? What is the impact on physiology of the capsule during development and are there links with alkaloid accumulation? The latter question is quite important, as it has been noted that solute concentrations affect both

cell turgor and the progression of biochemical pathways (Hrazdina and Jensen 1992, Tomos and Leigh 1999). It is apparent that this is an area that has received little attention and would be a worthwhile avenue of research.

6.5 Conclusions and recommendations

This study found that osmotic potential was significantly more negative in the latex of both capsules than in latex of the main stem and the base of the main stem (early in capsule development). The finding was deemed to be consistent with sink strength, capsule/stem latex differences, and turgor requirements of expanding laticifers.

This chapter brings to light several areas where further research would be advisable and fruitful, including an investigation of capsule osmotic potential during development; elucidation of the mechanisms of substance loading into laticifers; water fluxes and the mechanism of vessel refilling; and the links with alkaloid accumulation.

Much of the research in *P. somniferum* focuses on the localisation and activity of enzymes, proteins and alkaloid biosynthetic pathways (El-Ahmady and Nessler 2001, Facchini and De Luca 1995, Gerardy and Zenk 1993a, 1993b, Roberts *et al.* 1983), but there is a paucity of work on laticifer physiology - especially on osmotic potentials and water relations of laticifers in poppy (Pickard 2008). This represents an interesting and under-studied area of latex physiology that merits further investigation, as it would further elucidate the likelihood of *in toto* latex movement and clarify the noted relationships with adjacent cell types of the vascular bundle (Bird *et al.* 2003, Samanani *et al.* 2006, Weid *et al.* 2004). It would also inform on capsule development and physiology, and may potentially be important to alkaloid biosynthesis.

CHAPTER 7 General discussion and conclusions

Papaver somniferum L. is an important crop grown for both pharmaceutical and for culinary purposes. This species has received considerable research attention, with the agro-morphological aspects (Bhandari *et al.* 1997, Singh *et al.* 2000, Singh *et al.* 2003, Yadav *et al.* 2006), the localisation and activity of enzymes, proteins and alkaloid biosynthetic pathways being widely studied (El-Ahmady and Nessler 2001, Facchini and De Luca 1995, Gerardy and Zenk 1993a, 1993b, Roberts *et al.* 1983). Further, the ultrastructure of laticifers has also been thoroughly addressed (Nessler and Mahlberg 1977a, 1977b, 1978, 1979i, 1979ii, 1981) however; there are aspects of the laticifer system that have been overlooked. The work in this thesis sought to extend current research by examining two aspects of poppy laticifers that may contribute to alkaloid yield in capsules: the laticifer volume or capacity in the capsule and the supply of latex from stem to capsules.

7.1 Laticifer volume and timing of development

The volume of laticifers (per unit area) in a capsule, or latex capacity, was not found to be related to the morphine end-yield at commercial harvest, but it was determined that the volume was established early in capsule development. This major finding was consistent with the process of laticifer cell initiation and the development of vascular tissue. The articulated and anastomosing laticifers of poppy are found in the vascular bundle adjacent to phloem sieve cells, and differentiate from procambium almost immediately after the sieve elements arise in the seedling (Fahn 1989, Fairbairn and

Kapoor 1960, Nessler and Mahlberg 1978). Articulations are created through end wall dissolution, usually later in cell differentiation (Datta and Iqbal 1994, Nessler and Mahlberg 1977b), and laticifers do not increase in diameter later in development (Fairbairn and Kapoor 1960). This developmental process occurs in conjunction with surrounding cell development (Hagel *et al.* 2008), and explains why laticifer volume is set early in capsule growth, and why capacity does not change during development. Further growth would be proportional to that of surrounding cells in the capsule, and thus volume per unit area does not alter appreciably.

The early establishment of latex volume in capsules concurs with observations of latex development in organs of other species, such as the leaves of *Euphorbia pulcherrima* (Spilatro and Mahlberg 1986) and *Hoya australis* (Warnaar 1982), species with non-articulated laticifers and characterized by symplastic growth (Mahlberg 1993). Spilatro and Mahlberg (1986) proposed a link between the timing of latex development and the role in defense against herbivory, and although these species have a different mode of laticifer growth to those in poppy, the achievement of latex capacity early in capsule development may support the role of *P. somniferum* laticifers in the defense of young reproductive organs against herbivores.

7.2 Biomass allocation and resource supply

Although it was found that laticifer capacity was not a direct contributor to variability in crop alkaloid yield, a consideration of the early development of capacity, of the consistency of this trait across a crop, and of the close linear association with capsule

mass, has implications for the management of capsule mass in a *P. somniferum* crop. A number of factors have previously been identified that considerably increase capsule mass, including; level of irrigation (Chung 1987, Szabo *et al.* 2008), irrigation at certain stages, (Chung 1987, Tomar *et al.* 1990), nutrient application, (Bernáth and Tétényi 1986, Laughlin 1979, Laughlin *et al.* 1998), temperature (Bernáth and Tétényi 1981) and planting density (Bernáth and Tétényi 1982, Bhandari *et al.* 1989, Chung 1990, Muchova *et al.* 1993). It is important however, to consider the impact of these factors in more detail than at the broad crop or plant level, and to consider the maximisation of capsule mass for those capsules that contain the greatest concentration of alkaloids.

An investigation of the within-plant allocation of reproductive biomass demonstrated that greater than 80% of the capsule mass on a plant was allocated to the upper three positions, even on highly branched plants (section 3.4.1). Allocation to the upper capsules was described in terms of plant development; sink strength; and resource supply. The study revealed the sink strength and advantage of the sequential development of the upper capsules (section 3.5.2), a finding that concurs with resource allocation studies for other species (Buide 2008, Heuvelink 1996, Susko and Lovett-Doust 1999, Wolfe and Denton 2001). It was also suggested that the control of the production of lower reproductive structures contributed to maintaining capsule size at the upper positions. In terms of alkaloid accumulation, there was a position effect of capsules that was maintained at three of the four sites, an observation in agreement with previous research (Khanna and Gupta 1982, Loftus Hills 1945, Shukla *et al.* 2004). The within-plant position effect is therefore relatively consistent in terms of the development of capsule mass and alkaloid content, and indicates the influence of resource allocation to the stronger sinks of the upper capsules, the effect of the sequential development of

capsules (Bhandari *et al.* 1989, Ghiorghiță *et al.* 1990), and feedback mechanisms that may be operating within the *P. somniferum* plant relating to fruit and seed set (Wolfe and Denton 2001, Sadras and Ford Denison 2009). The variability evident in capsule alkaloid yields, however, indicates that environmental variables remain a major influence (section 3.5.4) and that although closely linked to the yield of capsule mass, improvements in alkaloid yield are less certain.

7.3 In situ latex development – the vesicle population

While the early chapters of the thesis investigated the structural contribution of capsule laticifers to alkaloid yield, the latter chapters addressed the functional aspects of laticifers. Although it was not possible to visualise the cells *in planta*, the studies of stem (peduncle) and capsule latex were able to describe laticifer contents in terms of their vesicle populations, patterns of vesicle development, and osmotic potential. The discovery of a significantly larger population of vesicles in capsule, the observed persistent steep gradient in both the PVV and the vesicle number, and the evident differences between positions in the pattern of latex development, indicated that *in situ* development of latex occurred, with localised synthesis of vesicles. *In situ* latex development concurs with the activity of the rapidly dividing tissues of the vascular cambium in the developing capsule, and also with the development of laticifers that articulate late in differentiation, where vesicles are commonly plentiful in laticifer cells prior to the development of perforations (Thureson-Klein 1970, Nessler and Mahlberg 1977a, 1977b). It is also in agreement with developmental differences between the latex of capsules and stems (Kamo and Mahlberg 1984, Bajpai *et al.* 2001, and results

herein; section 5.5.3 and 6.4); with the observed capsule sink strength (section 3.5.2) and with the rapid accumulation of capsule alkaloids. Several authors have presented results supporting the correlation between the differentiation of laticifers and the production of morphinan alkaloids (Kutchan *et al.* 1985, Rush *et al.* 1986, Samanani *et al.* 2006), and poppy capsules, with more numerous laticifers (and vesicles within) would support a rapid accumulation of these metabolites.

In situ production of latex and vesicles is also supportive of the notion that vesicles have a role in metabolic regulation (Facchini 2001), as they are present very early in laticifer development and are numerous in the actively developing tissue of the capsule. Further, local vesicles, with discrete subpopulations, would function to efficiently sequester alkaloids from the surrounding cytosol, enabling progression of biosynthetic pathways. This is in agreement with the notion that proximity creates efficient metabolic pathways (Alcantara *et al.* 2005, Hrazdina and Jensen 1992, Winkel 2004). The capacity of a heterogeneous population of translocated vesicles (presumably containing alkaloids) to efficiently participate in this process is unknown.

It is evident then, that there is much support for *in situ* vesicle production in the laticifers of *P. somniferum*. It also raises questions about the internal milieu of young capsule laticifers, especially considering the observation that the capsule latex had greater osmotic potential than the stem latex in early capsule development. While this could have been an artefact of sampling (refer section 6.4), the observations of plasmodesmatal connections and the identification of ABC transporter homologues (Facchini *et al.* 2005, Facchini *et al.* 2007) indicates that the physiology of laticifers, living cells at maturity, is intimately linked with other cells. Very little is known about

the physiology of laticifers in this regard, so future research in this area will be of great interest.

7.4 Latex translocation studies

The indication that bulk latex transport may not be a major contributor to capsule alkaloid accumulation requires a revisiting of the pioneering translocation studies. The early translocation studies that are commonly cited in support of bulk movement are unconvincing. The assertion that vesicles move upward in latex, and through *in toto* latex movement, resulted from the observed translocation of labelled morphine and tyrosine throughout the *P. somniferum* plant (Fairbairn and El-Masry 1967, Fairbairn *et al.* 1974). Presumably the authors based this conclusion on the premise that the applied labelled alkaloids were sequestered immediately and entirely to latex vesicles, a reasonable premise given that this is the fate of alkaloids in the plant (Fairbairn and Djote 1970, Fairbairn *et al.* 1974, Roberts *et al.* 1983). However, the same feeding cup method was used for each publication, yet close examination of the possibility of experimental contamination did not occur, nor was there the consideration that other cell types may have been responsible for translocation, and the surprisingly rapid rates of movement were not followed up.

With the benefit of recent research, it is becoming apparent that other cell types may be implicated in the biosynthesis of alkaloids (Bird *et al.* 2003, El-Ahmady and Nessler 2001, Samanani *et al.* 2006, Weid *et al.* 2004). It is often cited that alkaloids are sequestered to vesicles due to their cytotoxic effects (Alcantara *et al.* 2005, Facchini

2001, Hagel *et al.* 2008, Hendry 1986) but the involvement of other cells types discovered for *P. somniferum* brings this into question, especially in view of the work by Alcantara *et al.* 2005, who localised COR to the cytosol in dedifferentiated poppy cells. It could be posited that if morphinan alkaloids were highly cytotoxic to cells of *P. somniferum*, the biosynthetic pathway would be localised to laticifers only, as the ultimate site of storage. Further to this, as Yazaki (2006) points out, some species of alkaloid-producing plants appear unaffected (Waller and Nowacki 1978) or only moderately affected by their own metabolites (Sato *et al.* 1990), and it has been shown that in other species (e.g. *Senecio sp.*, *Nicotiana sp.*, *Lupinus sp.* and *Ruta sp.*), alkaloids are translocated in xylem or phloem (Baümel *et al.* 1995, Lee *et al.* 2007, Wink and Roberts 1998). A toxicity assay with cell suspension cultures could address the cytotoxicity of poppy alkaloids (Sato *et al.* 1990).

The possibility that alkaloids may be transported in other cell types allows for the reinterpretation of the previous results of translocation in *P. somniferum*, addressing the sites of detected (radio-labelled) morphine, the speed of movement and the observed movement in both directions.

Several studies have implied that the flow of radioactive substances occurred to the poppy capsule in the latex (Fairbairn *et al.* 1964, Fairbairn and El-Masry 1967, Fairbairn *et al.* 1974). The authors stated that there was no downward passage of latex and that the majority of substances accumulated in the pericarp and latex of the capsule (refer Table 1, Fairbairn and El-Masry 1967). In view of the coarse method in which labelled morphine was applied for both studies (to scraped tissue of the pedicel), and the possible tolerance of cells (dependent upon alkaloid concentration; Sato *et al.* 1990) an

alternative explanation is that partial transport of the labelled morphine occurred via the phloem. This is consistent with the rapid transport to the upper plant parts, and with the timing of appearance in the capsule tissue and ovules (which have no laticifer supply), just hours after loading the radioactive label (Fairbairn *et al.* 1974). Also, loading was performed at the pedicel of the plant and yet radioactive substances appeared lower down, in the leaves and roots (Fairbairn and El-Masry 1967). The authors had suggested that there was no *in toto* latex movement downward, and the vesicle studies presented herein described a strong upward gradient of vesicles, thus translocation most likely occurred in the phloem (which can transport substances in both directions). Further, in the 1967 study, the majority of fed morphine remained near the site of application, suggesting that it was actually absorbed into the laticifers, and it apparently remained at the same site for more than one day. The later study contradicted this, citing that latex movement effected rapid transport of alkaloid vesicles upward to the capsule at 5 cm/hr, notably a typical velocity for phloem (Knoblauch and van Bel 1998).

By allowing for partial incorporation of radioactive substances into laticifers and ascribing translocation to the phloem, a more precise analysis of the results and a more parsimonious explanation can be achieved. This alternative explanation raises questions about the actual sensitivity of *P. somniferum* tissues to their own metabolites, and also indicates that, as in other alkaloid producing species, alkaloids may be translocated in phloem (Hashimoto *et al.* 1991, Lee *et al.* 2007, Wink 1987).

7.5 Latex translocation – parsimony?

Although the nature of the development of the laticifer system and its component vesicle populations argue against the likelihood of translocation, and although the initial translocation studies for poppy may be reinterpreted, it is important to assess the parsimony of latex transport within laticifers in a broader context.

Several roles have been ascribed to the articulated laticifers of *P. somniferum*, the storage, synthesis and translocation of alkaloids, in addition to the function in protection against herbivores (Agrawal and Konno 2009, Facchini *et al.* 2007). Until recently the site of alkaloid biosynthesis had been ascribed entirely to laticifers, and the focus of research was on the localisation of the biosynthetic enzymes and alkaloids to particular fractions within the latex (Kutchan *et al.* 1986, Roberts *et al.* 1983). Combined with dedicated translocation studies, the observation that alkaloids accumulated in the capsule during development, and with the copious exudation on wounding, the articulated laticifers of poppy were also credited with the function of alkaloid transport (Annett 1920, Fairbairn and El-Masry 1967, Fairbairn *et al.* 1974, Nikonov 1958, Tookey *et al.* 1976).

Translocation appears to be further supported by the plant-level studies of enzyme location and activity. It is notable that in several studies, the relative expression of gene transcripts for key pathway enzymes was greater in the stem of poppy than in the flower or capsule (CYP80B1, COR; Huang and Kutchan 2000, 6OMT, CNMT, 4OMT, SAT; Samanani *et al.* 2006) and Bird *et al.* (2003) identified the greatest levels of COR in the roots. Given that the vesicle studies herein illustrated that the capsule contained a larger

population of vesicles than the stem (peduncle), and other literature cites greater alkaloid content in the capsule (Bajpai *et al.* 2001, Hofman and Menary 1979, Nikonov 1958), it would be reasonable to expect that relative gene expression (especially of COR) would be higher in the capsule. The apparent inconsistency may relate to the plant variety used, or to the timing of sampling from capsules, however in the study by Weid *et al.* (2004), all enzymes (except BBE) were in greatest concentration in the capsule, sampled less than two days after petal fall. Inconsistencies aside, greater expression in the lower parts of the plant may be taken to indicate that intermediates and certain alkaloids are translocated to the capsule.

It is telling, however, that for the majority of these enzymes their presence was detected (to varying degrees) in all plant organs. This appears counterintuitive to the argument for translocation in laticifers, in that the co-localisation of the site of synthesis and storage in every plant organ would, to some degree, negate the necessity for transport. In other species, long distance alkaloid translocation occurs when there is a spatial separation between the sites of synthesis and storage (Hashimoto *et al.* 1991), yet for poppy the spatial separation appears to occur at the cellular level (Bird *et al.* 2003, Samanani *et al.* 2006, Weid *et al.* 2004). Further, it is suggested that metabolic pathways are spatially organized and within close proximity to enhance efficiency (Hrazdina and Jensen 1992, Winkel 2004), therefore it appears inefficient to deliver intermediates or alkaloids to other organs of the plant when the site of storage is nearby. In *P. somniferum* there appears to be spatial separation at the cellular level for both the sanguinarine alkaloid pathway and the morphinan alkaloid pathway, and this is consistent with the roles of storage and defense. Sanguinarine is an anti-microbial alkaloid that accumulates in root tissue, and it appears that the storage of this alkaloid in

the root cortex serves to protect the stele from infection. The branch-point enzyme for the sanguinarine pathway is BBE, detected in stems, roots and leaves of poppy (Weid *et al.* 2004, Bird *et al.* 2003), but reported to localise to metaphloem and protoxylem (Facchini and De Luca 1995), vascular parenchyma in roots and in xylem parenchyma in petioles of young leaves (El-Ahmady and Nessler 2001), and sieve cells in seedlings (Samanani *et al.* 2006).

Sanguinarine has not been detected in laticifers (Cline and Coscia 1989, El-Ahmady and Nessler 2001) and Bock *et al.* (2002) suggested that the site of storage of benzophenanthridine alkaloids was actually in the root cortex idioblasts. This is a different mode of plant defense to that in the aerial organs and involves different cell types; therefore any translocation of BBE that may occur (Facchini 2001) is unlikely to occur in latex. As a consequence, other cell types are implicated in transport (if it occurs), namely, the existing vasculature. Transport in either xylem or phloem would be consistent with alkaloid translocation in other species (Hashimoto *et al.* 1991, Wink 1987).

The spatial separation of morphinan biosynthesis in aerial organs has implicated the vascular parenchyma (Weid *et al.* 2004), sieve cells and companion cells (Bird *et al.* 2003, Samanani *et al.* 2006), with authors suggesting that the majority, if not all, of biosynthesis occurs in cells other than laticifers. The level of laticifer involvement in alkaloid biosynthesis is contentious, but whether the end-stages of biosynthesis occur in laticifers (Weid *et al.* 2004, Decker *et al.* 2000) or only sequestration of alkaloids occurs (Bird *et al.* 2003, Samanani *et al.* 2006), either scenario is a strong case for the storage role of laticifers, but neither precludes the possibility of translocation. However,

considered in light of (1) the disparity in vesicle populations between the capsule and the stem, (2) the requisite movement of latex against a gradient of vesicles, (3) the dilution effect of stem latex on vesicle numbers and osmotic content, and (4) the volume of latex required to enter the capsule to effectively contribute to alkaloid accumulation, not to mention the development of articulated laticifers, then the occurrence of bulk latex movement that has previously been ascribed to the laticifer system is unlikely.

More probable is the role in protection against herbivory, widely proposed (Agrawal and Konno 2009) and consistent with the development of laticifers and their contents. Latex efflux after wounding occurs as a result of the positive pressure in the laticiferous system, and exudation acts as a physical and chemical deterrent to herbivores (Oppel *et al.* 2009, Wink 1993). The maintenance of turgor in these cells is critical to their function in exudation (section 6.4), and the diurnal fluctuations evident in latex water (Itenov *et al.* 1999) are presumably moderated by osmotic content (section 6.4), but there has been no elucidation of this.

Although the arguments against latex movement are convincing, if latex movement does occur in the laticifers the most probable mechanism would be through the creation of pressure differentials by transpiration. It may be that upward translocation of vesicles occurs through loss of latex water via capsule transpiration, creating a decrease in the capsule latex hydrostatic pressure, and resulting in a concomitant upward movement of latex as pressure equilibrates through the articulated cells (section 5.5.4). It would be unlikely that this mechanism would result in the translocation rates described by Fairbairn *et al.* (1974), especially as the walls of laticifers are elastic and would relax with the outward movement of water, maintaining internal pressure to a degree (Frey-

Wyssling 1952). Therefore, the probability that a 10% diurnal variation in latex water would be sufficient to translocate vesicles into the relaxed capsule laticifers (against a concentration gradient of a pre-existing vesicle population) remains to be investigated.

There are many factors that indicate that the translocation of substances in laticifers is unlikely, and that the alternate suggestion of transport within the established vascular system is reasonable. The hypothesis that alkaloid translocation may occur in the phloem of *P. somniferum* then, is worthy of consideration. The suggestion by Facchini (2001) that vesicles serve a role in metabolic regulation is consistent with the notion of transport in phloem, and consistent with the observed populations in stem and capsule latex. There is increasing evidence that the site of (parts of) alkaloid biosynthesis is localised to other cell types, therefore intermediates would be supplied to adjacent laticifers for final conversion and storage. The limited number of vesicles in stem latex may be an indication of the capability of the transport/storage infrastructure in stem laticifers. Thus, when intermediates are produced (in parenchyma or sieve cells) in quantities surplus to local laticifer capability, it may be posited that they are loaded into the phloem stream for translocation elsewhere. Further evidence is required to fully develop a hypothesis of the details of a mechanism for phloem transport including; the duration of plasmodesmatal connections, whether there are similar connections to adjacent parenchyma, and confirmation of the presence of transporters. It is also difficult to envisage how the entire process of synthesis, translocation, and the unloading of alkaloids may occur within sieve elements, so the further developments on the current debate of the site of alkaloid biosynthesis will be of great interest.

Conclusions

This thesis has revealed and discussed the relative contribution of the physical capacity of laticifers to the yield of alkaloids in poppy capsules, and the contribution to the accumulation of alkaloids in the capsule via the commonly proposed function of latex translocation.

It is concluded that, consistent with articulated laticifer cell initiation and development, the capsule laticifers of *P. somniferum* reach volumetric capacity (per unit area) early in capsule development, prior to the first week after flowering. As the vesicle population develops in concert with cell development and as vesicles are numerous often before perforations occur (Nessler and Mahlberg 1977a, 1977b), the majority of the observed population is therefore attributable to *in situ* production. The observed differences in the size and the development of the capsule and stem vesicle populations support this conclusion and indicate differential regulation between these organs. Further, the early development of both the volumetric capacity and the vesicle population in capsule laticifers enables the reticulated system to function in the protection of the developing capsule (Spilatro and Mahlberg 1986), and ensures that alkaloid accumulation can occur rapidly through metabolic regulation and the rapid sequestration of alkaloids.

Due to the consistent volume (per unit area) of laticifers in capsules they do not predict morphine yield at commercial harvest, however the linear relationship between alkaloid yield and capsule mass has management implications. Of particular interest for crop management is that the majority of within-plant reproductive biomass accumulates on the upper three capsules, through a complex interplay of plant development, sink

strength, resource supply and feedback mechanisms (tip senescence). It is recommended that a consideration of within-plant biomass allocation be incorporated in the management of crops, in order to fully optimise the production of dry mass of commercially important (high yielding) capsules.

The component vesicle populations of the capsule latex and the stem latex are predominantly derived from *in situ* production which, combined with; the turgor requirements of the network (to fulfil the role in protection through exudation), the presence of key enzymes in all organs, the spatial separation of synthesis at the cellular level and the requirement for metabolic efficiency, argues that the occurrence of bulk latex transport is doubtful. It is suggested that alkaloid translocation in *P. somniferum* may occur via the traditional vascular system, particularly phloem.

Research recommendations

Three main areas for future research are recommended:

- The deeper investigation of the vesicle populations in the latex of capsules and stems should be considered. The use of flow cytometry or other appropriate technologies would provide further insight into the biosynthetic capacity of the latex of different organs, identify relationships with alkaloid yield, and assess whether the population of vesicles in the stem latex could predict that of capsule latex.
- Further method development, testing and validation of techniques to assess pressure potentials in laticifers should be undertaken. There is a paucity of available research on laticifer physiology - especially on osmotic potentials and water relations of laticifers in poppy. This represents an interesting and under-studied area of latex physiology that; would further elucidate the likelihood of *in toto* latex movement; would clarify the noted relationships with adjacent cell types of the vascular bundle; would inform on capsule development and physiology and may potentially be important to alkaloid biosynthesis.
- In light of the evidence against bulk flow in laticifers, the implication of other cell types in the alkaloid biosynthetic pathway, the cited connections between laticifers and adjacent cells, and the recognition that alkaloids are translocated in the traditional vascular system (xylem and phloem) of other species, an examination of the role of these cell types (especially phloem) in the translocation of the enzymes/precursors of the alkaloid biosynthetic pathway in *P. somniferum* has merit.

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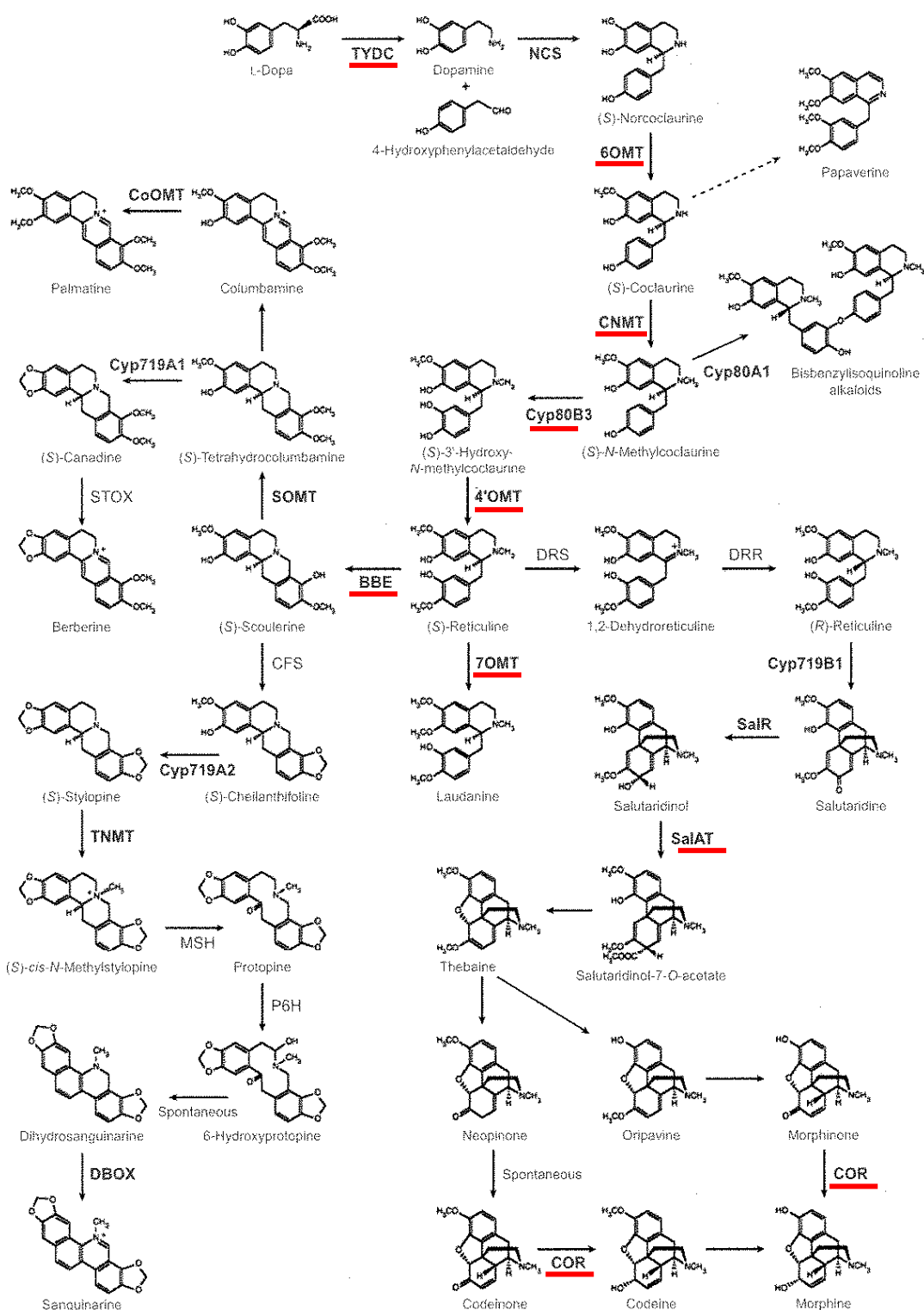
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**Figure 2**

Biosynthesis of the benzylisoquinoline alkaloids berberine, morphine, and sanguinarine. Enzymes for which corresponding molecular clones have been isolated are shown in bold. Abbreviations: 4_OMT, 3'-hydroxy-N-methylcoclaurine 4_-O-methyltransferase; 6OMT, norcoclaurine 6-O-methyltransferase; 7OMT, reticuline 7-O-methyltransferase; BBE, berberine bridge enzyme; CFS, cheilanthifoline synthase; CNMT, coclaurine N-methyltransferase; CoOMT, columbamine O-methyltransferase; COR, codeinone reductase; Cyp719A1, canadine synthase; Cyp719A2, stylopine synthase; Cyp719B1, salutaridine synthase; Cyp80A1, berbaminine synthase; Cyp80B3, N-methylcoclaurine 3'-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; DRR, 1,2-dehydroreticuline reductase; DRS, 1,2-dehydroreticuline synthase; MSH, N-methylstylopine 14-hydroxylase; NCS, norcoclaurine synthase; P6H, protopine 6-hydroxylase; SAT, salutaridinol 7-O-acetyltransferase; SalR, salutaridine:NADPH 7-oxidoreductase; SOMT, scoulerine 9-O-methyltransferase; STOX, (S)-tetrahydroxyprotoberberine oxidase; TNMT, tetrahydroprotoberberine cis-N-methyltransferase; TYDC, tyrosine decarboxylase.

Adapted from Zeigler and Facchini (2008). NB: CYP80B3 step = CYP80B1.

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